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(54) **Recombinant antibodies specific for a growth factor receptor.**

(57) The invention concerns recombinant antibodies directed to the extracellular domain of the human growth factor receptor c-erbB-2 comprising a light chain variable domain and a heavy chain variable domain of a monoclonal antibody, monoclonal antibodies directed to c-erbB-2 themselves, a method of manufacture of said recombinant antibodies and said monoclonal antibodies, hybridoma cells secreting said monoclonal antibodies, a method of manufacture of said hybridoma cells, DNA coding for the heavy chain variable domain, for the light chain variable domain and for the recombinant antibody, a method of manufacture of said DNA, hybrid vectors suitable for expression of said DNA, host cells transformed with said DNA, and the use of said recombinant antibodies and said monoclonal antibodies in the diagnosis and treatment of tumors.

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Background of the invention

Growth factors and their receptors ere involved in the regulation of cell proliferation, and they also seem to play a role in tumor growth. The c-erbB-2 growth factor receptor protein, a protein of the membrane receptor protein tyrosine kinase family (A. Ullrich & J.Schlessinger, Cell 61: 203-212, 1990), is found In human breast tumors and human ovarian cercinomes. Amplification of the c-erbB-2 gene end over-expression of the protein appears to correlate wlth poor prognosis for tumor patients. Thus the c-erbB-2 protein has potential, both as e diegnostic marker and as a target for cancer therapy. Sequence analysis reveals that c-erbB-2, also called HER2, a glycoprotein of 185 kilo-Dalton (gp 185), is identical or closely releted to the humen analog of the neu oncogene (A.L.Schechter et al., Science 229: 976-978, 1985) and shows considerable sequence homology to the human epidermel growth factor (EGF) receptor.

Of particular interest in tumor diegnosis and therapy are antibodies directed to tumor markers. Polyclonal antibodies may be obtained from the serum of mammals immunized with the antigen, i.e. the tumor marker. The development of hybridoma technology made it possible to generate continuous cell lines, in particular murine hybridomas, producing monoclonel antibodies of the desired specificity. Murine monoclonal antibodies directed to c-erbB-2 are known and are described, for example, by S.J.McKenzie et el., Oncogene 4: 543-548, 1989; R.M.Hudzlak et al., Moleculer and Celluler Biology 9: 1165-1172, 1989; Internetionel Patent Application WO 89/06692 (Genentech); and Japanese Petent Application Kokai 02-150 293 (Ajinomoto KK).

A mejor limitation in the use of murine-derived monoclonal antibodies as in vivo diagnostic and therapeutic agents is their immunogenicity es foreign proteins, their rether long persistence in the circulation, end the formation of dameging immune complexes. On the other hend, the treetment with humen monoclonel entibodies is also limited since human hybridoma cell lines are hard to prepare, generally unstable, and do not produce monoclonal antibodies of appropriate specificity in sufficient quantities end at reasoneble costs. In principle, the in vitro use of murine monoclonal antibodies is without limitation. However, production costs of monoclonal antibodies and, depending on the type of Immunoassey used, the need for attaching a detectable marker to the antibody make it desirable to find more economic alternatives to regular murine monoclonal antibodies.

A promising alternative is the modification of immunoglobulin genes In order to tallor antibodies for particular diagnostic and therapeutic tasks. Due to the fact that the variable region and each of the constant region domains of immunoglobulin molecules are encoded in separete exons with their own splice sites, recombinent DNA techniques can be used to isolate different parts of cloned immunoglobulin genes and ligate them to parts of other immunoglobulins or to effector molecules. The reconstructed genes are expressed by appropriate transformed continuous cell lines. Murine entibodies can, for example, be converted into "humanized" antibodies by exchanging murine constant domain exons for human immunoglobulin constant domain exons, thus generating chimeric antibodies with murine antibody-combining sites and human constant domains. The chimeric antibodies retain the antigen specificity determined by the murine variable domains, but also exhibit human effector functions (such as complement binding, stimulation of phagocytosis, triggering of granule release by mast cells) determined by the carboxy-terminal constant domain segments of the heavy chain polypeptides. An even more sophisticated technique in tailoring antibodies described in European Patent Application 0 239 400 exchanges also other feirly conserved domeins, the so-called framework regions (FRs), within the murine variable domains for corresponding framework regions from humen entibodies or for other human protein sequences. Such an antibody should be even less immunogenic in men since the only parts derived from a murine antibody are those hypervariable regions which define a particular specificity for an antigen, the so-called complementarity determining regions (CDRs).

Furthermore, fusion proteins different from immunoglobulins may be formed, e.g. single-chain antibodies, which retain the specificity and binding properties of the starting murine monoclonal antibody, but have otherwise novel properties derived from the non-immunoglobulin part of the fusion protein. The smallest domein of a monoclonal antibody which can bind to the antigen is the so-called Fv fragment which consists of the variable domains of the heavy and light cheins. Fv fragments are difficult to prepare by proteolytic techniques since the corresponding variable domains tend to dissociate upon dilution. Fv molecules constructed by joining the variable domains of the heavy and light chains via a short peptide linker, also called single-chain antigen binding proteins, bind to an antigen with similar characteristics as the originel monoclonal antibody (R.E. Bird et al., Science 242: 423-426, 1988; J.S.Huston et al., Proc. Natl. Acad. Sci. USA 85: 5879-5883, 1988; and International Patent Application WO 89/09825 (Celltech)). Fv encoding genes can, in principle, be linked to genes encoding effector molecules by recombinent gene technology. It is known, for example, that Fv encoding gene sequences can be linked to a gene encoding a portion of the Pseudomonas exotoxin A gene (V.K. Cheudhary et al., Nature 339: 394-397, 1989; and International Patent Application WO 89/11533 (I. Pastan et al.)).

Object of the invention

It is an object of this invention to provide recombinant antibodies directed to the extracellular domain of the human growth factor receptor c-erbB-2 comprising a light chain variable domain and a heavy chain variable domain of a monoclonal antibody, monoclonal antibodies directed to c-erbB-2 themselves, a method of manufacture of said recombinant antibodies and said monoclonal antibodies, hybridoma cells secreting said monoclonal antibodies, a method of manufacture of said hybridoma cells, DNA coding for the heavy chain variable domain, for the light chain variable domain and for the recombinant antibody, a method of manufacture of said DNA, hybrid vectors suitable for expression of said DNA, host cells transformed with said DNA, and the use of said recombinant antibodies and said monoclonal antibodies in the diagnosis and treatment of tumors.

Detailed description of the invention

The invention concerns a recombinant antibody directed to the extracellular domain of the growth factor receptor c-erbB-2, a human glycoprotein of 185 kilo-Dalton (gp185), comprising a heavy chain variable domain and a light chain variable domain of a monoclonal antibody.

Such a recombinant antibody may be a chimeric antibody consisting, for example, of a mouse heavy chain variable domain with the specificity for c-erbB-2 and a human heavy chain constant domain α, γ, δ, ε, or μ, preferably γ, such as $\gamma 1$ or $\gamma 4$, and of a mouse light chain variable domain with the specificity for c-erbB-2 and a human light chain constant domain κ or λ, preferably κ, all assembled to give a functional antibody.

The preferred recombinant antibody of the invention is a single-chain antibody wherein the heavy chain viable domain and the light chain variable domain are linked by way of a spacer group, preferably a peptide. Most preferred is a single-chain antibody wherein the heavy chain variable domain is located at the N-terminus of the recombinant antibody. The single-chain recombinant antibody may further comprise an effector molecule and/or signal sequences facilitating the processing of the antibody by the host cell in which it is prepared. Effector molecules considered are those useful for diagnostic or therapeutic purposes, for example enzymes causing a detectable reaction, e.g. phosphatase, such as alkaline phosphatase from E.coli or mamalian alkaline phosphatase, e.g. bovine alkaline phosphatase, horseradish peroxidase, β-D-galactosidase, glucose oxidase, glucoamylase, carbonic anhydrase, acetylcholinesterase, lysozyme, malate dehydrogenase or glucose-6-phosphate, a peptide having particular binding properties, e.g. streptavidin from Streptomyces avidinii strongly binding to biotin, or enzymes, toxins or other drugs attacking the cells to which the antibody is bound, e.g. a protease, a cytolysin or an exotoxin, for example ricin A, diphtheria toxin A, or Pseudomonas exotoxin. In the following a single-chain recombinant antibody further comprising an effector molecule is referred to as fusion protein or intended to be within the meaning of the terms "single chain (recombinant) antibody " or "recombinant antibody", if appropriate.

The term effector molecule also includes biologically active variants of the above-mentioned proteins, e.g. variants produced from a DNA which has been subjected to In vitro mutagenesis, with the provision that the protein encoded by said DNA retains the biological activity of the native protein. Such modifications may consist in an addition, exchange or deletion of amino acids, the latter resulting in shortened variants. For example, an enzyme, such as phosphatase, may be prepared from a DNA which has been modified to facilitate the cloning of the encoding gene, or an exotoxin, such as Pseudomonas exotoxin, may be prepared from a DNA which has been mutated to delete the cell binding domain.

The recombinant antibodies of the invention are tested for their specificity to the extracellular domain of c-erbB-2, for example by immunofluorescent staining of cells expressing high levels of c-erbB-2, by immunoblotting either directly or by way of immunoprecipitation and protein blotting of the immunocomplexes, or by another immunoassay such as a binding, crossinhibition or competition radio- or enzyme immunoassay.

The variable domain of an antibody heavy or light chain consists of so-called framework regions (FRs), which are fairly conserved in antibodies with different specificities, and of hypervariable regions also called complementarity determining regions (CDRs), which are typical for a particular specificity.

Preferred recombinant antibodies of the invention are those wherein the heavy chain variable domain comprises a polypeptide of the formula

$$FR_1\text{-}CDR_{1H}\text{-}FR_2\text{-}CDR_{2H}\text{-}FR_3\text{-}CDR_{3H}\text{-}FR_4 \qquad (I)$$

wherein FR_1 is a polypeptide residue comprising at least 25-29, preferably 25-33 naturally occurring amino acids, FR_2 is a polypeptide residue comprising 12-16 naturally occurring amino acids, FR_3 is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR_4 is a polypeptide residue comprising at least 6-10, preferably 6-13 naturally occurring amino acids, CDR_{1H} is a polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:4, CDR_{2H} is a polypeptide residue of the amino acid sequence 51 to 67 of SEQ ID NO:4, and CDR_{3H} is a polypeptide residue of the amino acid sequence 100 to 109 of SEQ ID NO:4, or, CDR_{1H} is a

polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:8, CDR_{2H} is a polypeptide residue of the emino ecid sequence 51 to 67 of SEQ ID NO:8, and CDR_{3H} is a polypeptide residue of the amino acid sequence 100 to 110 of SEQ ID NO:8, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges. These particular complementarity determining regions are Asn-Tyr-Gly-Met-Asn (CDR_{1H}), Trp-Ile-Asn-Thr-Ser-Thr-Gly-Glu-Ser-Thr-Phe-Ala-Asp-Asp-Phe-Lys-Gly (CDR_{2H}), and Trp-Glu-Val-Tyr-His-Gly-Tyr-Val-Pro-Tyr (CDR_{3H}) according to SEQ. ID NO: 4, or Ser-Tyr-Trp-Met-Asn (CDR_{1H}), Met-Ile-Asp-Pro-Ser-Asp-Ser-Glu-Thr-Gln-Tyr-Asn-Gln-Met-Phe-Lys-Asp (CDR_{2H}) and Gly-Gly-Ala-Ser-Gly-Asp-Trp-Tyr-Phe-Asp-Val (CDR_{3H}) according to SEQ. ID NO:8.

Especially preferred ere recombinent antibodies comprising a heavy chain variable domain of formule I, wherein the polypeptide residues of the framework regions FR_1, FR_2, FR_3 and FR_4 are those preferably occurring in memmalian, especielly murine or human, antibodies.

In a first embodiment of the invention, most preferred are recombinant antibodies with a heavy chain veriable domain comprising a polypeptide of the amino acid sequence 2 to 120, of SEQ ID NO:4, wherein optionelly one or more, e.g. 1, 2, 3 or 4, single amino acids within the amino acid sequences 2 to 31 (FR_1), 37 to 50 (FR_2), 68 to 99 (FR_3), and/or 110 to 120 (FR_4), are repleced by other amino acids or deleted, and whereln the emino acid Cys mey be in the oxidized state forming S-S-bridges, in particular the recombinant antibodies with a heavy chain variable domain comprising e polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:4, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

In a second embodiment of the invention, most preferred are recombinant antibodies wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 121, of SEQ ID NO: 8, wherein optionally one or more, e.g. 1, 2, 3 or 4, single amino acids within the amino ecid sequences 2 to 31 (FR_1), 37 to 50 (FR_2), 68 to 99 (FR_3), and/or 111 to 121 (FR_4), are replaced by other emino acids or deleted, end wherein the amino acid Cys may be in the oxidized state forming S-S-bridges, in particular the recombinant antibodies with a heavy chain variable domain comprising a polypeptide of the amino acid sequence 2 to 121 of SEQ ID NO: 8, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

For example, a hydrophobic amino acid within the framework regions may be replaced by another amino acid, preferably elso a hydrophobic emino ecid, e.g. a homologous amino acid, replaced by two emino acids, or deleted. Likewise, a hydrophilic amino acid within the framework region may be replaced by enother amino acid, two amino acids or deleted, whereby replacing emino ecids preferably maintain the hydrogen bond structure of the corresponding framework region.

Likewise preferred recombinant antibodies of the invention are those wherein the light chain varieble domain comprises a polypeptide of the formula

$$FR_6\text{-}CDR_{1L}\text{-}FR_7\text{-}CDR_{2L}\text{-}FR_8\text{-}CDR_{3L}\text{-}FR_9 \qquad (II)$$

wherein FR_6 is a polypeptide residue comprising naturally occurring amino acids, preferably 19-25, especially 19-23 naturally occurring amino acids, FR_7 is a polypeptide residue comprising 13-17 naturally occurring amino acids, FR_8 is a polypeptide residue comprising 30-34 naturally occurring emino acids, FR_9 is a polypeptide residue comprising naturally occurring amino ecids, particularly 7-11 naturelly occurring amino acids, and CDR_{1L} is a polypeptide residue of the amino acid sequence 159 to 169 of SEQ ID NO:4, CDR_{2L} is a polypeptide residue of the amino acid sequence 185 to 191 of SEQ ID NO:4, and CDR_{3L} is e polypeptide residue of the emino acid sequence 224 to 232 of SEQ ID NO:4, or CDR_{1L} is a polypeptide residue of the amino acid sequence 160 to 170 of SEQ ID NO:8, CDR_{2L} is a polypeptide residue of the amino acid sequence 186 to 192 of SEQ ID NO:8, and CDR_{3L} is a polypeptide residue of the amino acid sequence 225 to 232 of SEQ ID NO:8, and wherein the emino acid Cys may be in the oxidized stete forming S-S-bridges. These particular complementarity determining regions ere Lys-Ala-Ser-Gln-Asp-Val-Tyr-Asn-Ala-Val-Ala (CDR_{1L}), Ser-Ala-Ser-Ser-Arg-Tyr-Thr (CDR_{2L}), and Gln-Gln-His-Phe-Arg-Thr-Pro-Phe-Thr (CDR_{3L}) eccording to SEQ ID No:4, or Lys-Ala-Ser-Gln-Asp-Ile-Lys-Lys-Tyr-Ile-Ala (CDR_{1L}), Tyr-Thr-Ser-Vel-Leu-Gln-Pro (CDR_{2L}) and Leu-His-Tyr-Asp-Tyr-Leu-Tyr-Thr (CDR_{3L}) according to SEQ ID No. 8.

Especially preferred are recombinant antibodies comprising a light chain variable domain of formula II, wherein the polypeptide residues of the framework regions FR_6, FR_8, FR_7 end FR_8 are those preferebly occurring in mammalian, especially murine or human, antibodies.

In one embodiment of the invention, most preferred are recombinant antibodies wherein the light chain variable domain comprises a polypeptide of the amino ecid sequence 136 to 241 of SEQ ID NO:4, wherein optionelly one or more, e.g. 1, 2, 3 or 4, single amino acids within the amino acid sequences 136 to 158 (FR_6), 170 to 184 (FR_7), 192 to 223 (FR_8), and/or 233 to 241 (FR_9) are repleced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges, in particular the recombinant antibodies with a light chain veriable domain comprising a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:4, whereln the emino acid Cys may be in the oxidized state forming S-S-bridges.

In a second embodiment of the Invention, most preferred are recombinant antibodies wherein the light chain

variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:8, wherein optionally one or more, e.g. 1, 2, 3 or 4 single amino acids within the amino acid sequences 137 to 159 (FR_8), 171 to 185 (FR_7), 193 to 224 (FR_8), and/or 233 to 241 (FR_9) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges, in particular the recombinant antibody wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:8, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

For example, amino acids within the framework regions may be replaced by other amino acids or deleted as detailed above for the heavy chain.

Especially preferred is a single-chain recombinant antibody wherein the heavy chain variable domain and the light chain variable domain are linked by way of a spacer group consisting of 10 to 30, e.g. around 15, amino acids, in particular a single-chain recombinant antibody comprising a polypeptide of the formula

$$FR_1\text{-}CDR_{1H}\text{-}FR_2\text{-}CDR_{2H}\text{-}FR_3\text{-}CDR_{3H}\text{-}FR_4\text{-}Sp\text{-}FR_6\text{-}CDR_{1L}\text{-}FR_7\text{-}CDR_{2L}\text{-}FR_8\text{-}CDR_{3L}\text{-}FR_9 \quad (III),$$

wherein FR_1, CDR_{1H}, FR_2, CDR_{2H}, FR_3, CDR_{3H}, FR_4, FR_6, CDR_{1L}, FR_7, CDR_{2L}, FR_8, CDR_{3L} and FR_9 have the meanings as mentioned before and Sp is a peptide spacer consisting of about 10 to 30, e.g. around 15, amino acids; and wherein the heavy chain or the light chain variable domain is further connected to an effector molecule, e.g. an enzyme, such as phosphatase, particularly alkaline phosphatase, or a toxin, such as Pseudomonas exotoxin, or a variant thereof. Preferably, the effector molecule is connected to the light chain variable domain, optionally via a peptide spacer consisting of one or more, e.g. 1-10 amino acids.

These fusion proteins comprising a single-chain recombinant antibody and an effector molecule optionally comprise another peptide, e.g. a peptide facilitating purification, in particular a peptide being an epitope against which an antibody is available, such as the FLAG peptide. Purification, e.g. by means of affinity chromatography, of a fusion protein comprising such a peptide is advantageous e.g. in that it may be faster, more specific and/or gentler. The peptide may be placed at the N-terminus of the fusion protein, in between the recombinant antibody and the effector molecule, or at the C-terminus of the fusion protein. Preferably, it is located at the N-terminus or at the C-terminus, in particular at the N-terminus. Preferably, these constructs also contain a cleavage site, so that the fusion protein can be liberated therefrom, either by enzymatic cleavage, e.g. by enterokinase or by Factor Xa, or by the chemical methods known in the art. Furthermore these constructs may comprise a peptide spacer consisting of one or more, e.g. 1 to 10, in particular about 2 amino acids, said spacer facilitating the linkage of the above-mentioned peptide and/or the cleavage site to the recombinant antibody. The cleavage site is placed in such a way that the fusion protein comprising the recombinant antibody and the effector molecule can be easily liberated, if desired, preferably in vitro. For example, in the protein construct comprising the fusion protein designated Fv(FRP5)-ETA (cf. SEQ. ID NO: 10), the FLAG peptide and an enterokinase cleavage site are linked to a spacer and placed in front of the Fv heavy chain/light chain variable domain and exotoxin A fusion protein. If desired, the FLAG peptide can be cleaved off by enterokinase, preferably after affinity purification of the protein, yielding a fusion protein comprising the single-chain antibody Fv(FRP5) and exotoxin A.

Most preferred is a single-chain recombinant antibody wherein the heavy chain variable domain and the light chain variable domain are derived from a mouse monoclonal antibody directed to the extracellular domain of the growth factor receptor c-erbB-2, e.g. derived from the mouse monoclonal antibodies FRP5, FSP16, FWP51 or FSP77, particularly from the mouse monoclonal antibodies FRP5 or FWP51. Likewise preferred is a single-chain recombinant antibody wherein the spacer group linking the light chain and the heavy chain variable domains is a polypeptide comprising about 15 amino acids selected from glycine and serine, in particular wherein the spacer group is the 15 amino acid polypeptide consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser.

Especially preferred is a single-chain antibody comprising the heavy chain variable domain of a mouse monoclonal antibody selected from the group consisting of FRP5, FSP16, FWP51 and FSP77, the 15 amino acid spacer group consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser, the light chain variable domain of a mouse monoclonal antibody selected from the group consisting of FRP5, FSP16, FWP51 and FSP77 and an enzyme, for example a phosphatase such as the alkaline phosphatase phoA, or an exotoxin such as Pseudomonas exotoxin, or a variant thereof.

Particularly preferred is the particular single-chain recombinant antibody designated Fv(FRP5)-phoA comprising a polypeptide of the amino acid sequence 2 to 690 of SEQ ID NO: 5.

Likewise preferred is a single-chain recombinant antibody comprising a peptide facilitating purification, a cleavage site and a particular single-chain recombinant antibody selected from the group consisting of Fv(FRP5)-ETA and Fv(FWP51)-ETA, in particular a single-chain recombinant antibody comprising a polypeptide selected from the group consisting of a polypeptide of the amino acid sequence -10 to 606 of SEQ. ID NO: 10 and of a polypeptide of the amino acid sequence -10 to 606 of SEQ. ID NO: 11, said protein being subjected to in vitro cleavage by enterokinase, if desired.

Particularly preferred is a single-chain recombinant antibody comprising a protein selected from the group consisting of a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO: 10 and a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO: 11.

The invention further concerns the mouse monoclonal antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2 and designated FRP5, FSP16, FSP77, and FWP51, which are secreted by the hybridoma cell lines FRP5, FSP16, FSP77, and FWP51, respectively. Most preferred are the mouse monoclonal antibodies designated FRP5 and FWP51.

The invention further concerns a method of manufacture of the recombinant antibodies and of the mouse monoclonal antibodies of the invention. The antibodies are prepared by processes that are known per se, characterized in that host cells or hybridoma cells as defined further below producing such antibodies are multiplied in vitro or in vivo and, when required, the obtained antibodies are isolated. For example, the recombinant antibodies of the invention can be prepared by recombinant DNA techniques comprising culturing a transformed host under conditions which allow expression thereof and isolating said antibody.

More specifically, the present invention also relates to a process for the production of a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer comprising culturing a host, e.g. E. coli, which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter and a DNA coding for said protein which DNA is controlled by said promoter, and isolating said protein.

In particular, the present invention relates to a process for the production of a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, and a fusion protein optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer comprising culturing a host, e.g. E. coli, which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding said protein, and isolating said protein.

Multiplication of hybridoma cells or mammalian host cells in vitro is carried out in suitable culture media, which are the customary standard culture media, for example Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium, optionally replenished by a mammalian serum, e.g. fetal calf serum, or trace elements and growth sustaining supplements, e.g feeder cells such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages, 2-aminoethanol, insulin, transferrin, low density lipoprotein, oleic acid, or the like. Multiplication of host cells which are bacterial cells or yeast cells is likewise carried out in suitable culture media known in the art, for example for bacteria in medium LB, NZCYM, NZYM, NZM, Terrific Broth, SOB, SOC, 2 x YT, or M9 Minimal Medium, and for yeast in medium YPD, YEPD, Minimal Medium, or Complete Minimal Dropout Medium.

In vitro production provides relatively pure antibody preparations and allows scale-up to give large amounts of the desired antibodies. Techniques for bacterial cell, yeast or mammalian cell cultivation are known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapped cell culture, e.g. in hollow fibres, microcapsules, on agarose microbeads or ceramic cartridges.

Large quantities of the desired antibodies can also be obtained by multiplying mammalian cells in vivo. For this purpose, hybridoma cells producing the desired antibodies are injected into histocompatible mammals to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially mineral oils such as pristane (tetramethyl-pentadecane), prior to the injection. After one to three weeks, the antibodies are isolated from the body fluids of those mammals. For example, hybridoma calls obtained by fusion of suitable myeloma cells with antibody-producing spleen cells from Balb/c mice, or transfected cells derived from hybridoma cell line Sp2/0 that produce the desired antibodies are injected intraperitoneally into Balb/c mice optionally pre-treated with pristane, and, after one to two weeks, ascitic fluid is taken from the animals.

The cell culture supernatants are screened for the desired antibodies, preferentially by immunofluorescent staining of cells expressing c-erbB-2, by immunoblotting, by an enzyme immunoassay, e.g. a sandwich assay or a dot-assay, or a radioimmunoassay.

For isolation of the antibodies, the immunoglobulins in the culture supernatants or in the ascitic fluid may be concentrated, e.g. by precipitation with ammonium sulphate, dialysis against hygroscopic material such as polyethylene glycol, filtration through selective membranes, or the like. If necessary and/or desired, the antibodies are purified by the customary chromatography methods, for example gel filtration, ion-exchange chromatography, chromatography over DEAE-cellulose and/or (immuno-)affinity chromatography, e.g. affinity

chromatography with c-erbB-2 protein or with Protein-A.

The invention further concerns hybridoma cells secreting the monoclonal antibodies of the invention, in particular the hybridoma cell lines FRP5, FSP16, FSP77, and FWP51 deposited under the Budapest Treaty on November 21, 1990 at the European Collection of Animal Cell Cultures (ECACC) in Porton Down, Salibury, UK, under the accession numbers 90112115, 90112116, 90112117, and 90112118, respectively. Most preferred is the hybridoma cell line designated FRP5, ECACC number 90112115 or the hybridoma cell line designated FWP51, ECACC number 90112118. The preferred hybridoma cells of the invention are genetically stable, secrete monoclonal antibodies of the invention of the desired specificity and can be activated from deep-frozen cultures by thawing and recloning.

The invention also concerns a process for the preparation of a hybridoma cell line secreting monoclonal antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2, characterized in that a suitable mammal, for example a Balb/c mouse, is immunized with purified c-erbB-2 protein, an antigenic carrier containing purified c-erbB-2 or with cells bearing growth factor receptor c-erbB-2, antibody-producing cells of the immunized mammal are fused with cells of a suitable myeloma cell line, the hybrid cells obtained in the fusion are cloned, and cell clones secreting the desired antibodies are selected. For example spleen cells of Balb/c mice immunized with cells bearing c-erbB-2 are fused with cells of the myeloma cell line PAI or the myeloma cell line Sp2/0-Ag 14, the obtained hybrid cells are screened for secretion of the desired antibodies, and positive hybridoma cells are cloned.

Preferred is a process for the preparation of a hybridoma cell line, characterized in that Balb/c mice are immunized by injecting subcutaneously and/or intraperitoneally between 10^7 and 10^8 cells of the human breast tumor cell line SKBR3 containing a suitable adjuvant several times, e.g. four to six times, over several months, e.g. between two and four months, and spleen cells from the immunized mice are taken two to four days after the last injection and fused with cells of the myeloma cell line PAI in the presence of a fusion promoter, preferably polyethylene glycol. Preferably the myeloma cells are fused with a three- to twentyfold excess of spleen cells from the immunized mice in a solution containing about 30 % to about 50 % polyethylene glycol of a molecular weight around 4000. After the fusion the cells are expanded in suitable culture media as described hereinbefore, supplemented with a selection medium, for example HAT medium, at regular intervals in order to prevent normal myeloma cells from overgrowing the desired hybridoma cells.

The invention also concerns recombinant DNAs comprising an insert coding for a heavy chain murine variable domain and/or for a light chain murine variable domain of antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2 as described hereinbefore. By definition such DNAs comprise coding single stranded DNAs, double stranded DNAs consisting of said coding DNAs and of complementary DNAs thereto, or these complementary (single stranded) DNAs themselves.

Furthermore, DNA encoding a heavy chain murine variable domain and/or for a light chain murine variable domain of antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2 can be enzymatically or chemically synthezised DNA having the authentic DNA sequence coding for a heavy chain murine variable domain and/or for the light chain murine variable domain, or a mutant therof. A mutant of the authentic DNA is a DNA encoding a heavy chain murine variable domain and/or a light chain murine variable domain of the above-mentioned antibodies in which one or more amino acids are deleted or exchanged with one or more other amino acids. Preferably said modification(s) are outside the CDRs of the heavy chain murine variable domain and/or of the light chain murine variable domain of the antibody. Such a mutant DNA is also intended to be a silent mutant wherein one or more nucleotides are replaced by other nucleotides with the new codons coding for the same amino acid(s). Such a mutant sequence is also a degenerated sequence. Degenerated sequences are degenerated within the meaning of the genetic code in that an unlimited number of nucleotides are replaced by other nucleotides without resulting in a change of the amino acid sequence originally encoded. Such degenerated sequences may be useful due to their different restriction sites and/or frequency of particular codons which are preferred by the specific host, particularly E. coli, to obtain an optimal expression of the heavy chain murine variable domain and/or a light chain murine variable domain.

The term mutant is intended to include a DNA mutant obtained by in vitro mutagenesis of the authentic DNA according to methods known in the art.

The invention relates to a recombinant DNA comprising an insert coding for a heavy chain murine variable domain of a monoclonal antibody selected from the group consisting of the antibodies FRP5, FSP16, FSP77 and FWP51, or coding for an amino acid sequence homologous to said heavy chain variable domain.

In particular, the invention concerns a recombinant DNA comprising an insert coding for a heavy chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell lines FRP5, FSP16, FSP77 or FWP51, or which is homologous to genomic DNA of said cell lines and codes for an amino acid sequence homologous to the heavy chain variable domain of monoclonal antibodies FRP5, FSP16, FSP77 or FWP51. Especially preferred is a recombinant DNA comprising an insert coding for a heavy chain murine

variable domain, which originates from genomic DNA or mRNA of the hybridoma cell line FRP5, or which is homologous to genomic DNA of said cell line and codes for an amino acid sequence homologous to the heavy chain variable domain of monoclonal antibody FRP5; or a recombinant DNA comprising an insert coding for a heavy chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell line FWP51, or which is homologous to genomic DNA of said cell line and codes for an amino acid sequence homologous to the heavy chain variable domain of monoclonal antibody FWP51

Preferred is a recombinant DNA comprising an insert coding for the polypeptide of formula I, wherein FR_1, FR_2, FR_3, FR_4, CDR_{1H}, CDR_{2H}, and CDR_{3H} have the meanings as mentioned hereinbefore, optionally further containing introns. Especially preferred is a recombinant DNA coding for the polypeptide of formula I comprising inserts coding for murine or human framework regions FR_1, FR_2, FR_3 and FR_4, and inserts coding for complementarity determining regions of the DNA sequence 99 to 113 (CDR_{1H}), the DNA sequence 156 to 206 (CDR_{2H}), and the DNA sequence 303 to 332 (CDR_{3H}) of SEQ ID NO:4 or coding for complementarity determining regions of the DNA sequence 99 to 113 (CDR_{1H}), the DNA sequence 156 to 206 (CDR_{2H}), and the DNA sequence 303 to 335 (CDR_{3H}) of SEQ ID NO:8. Most preferred is a DNA comprising an insert of the DNA sequence 9 to 365 of SEQ ID NO:4, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 9 to 365 of SEQ ID NO:4. Likewise preferred is a DNA comprising an insert of the DNA sequence 9 to 368 of SEQ ID NO:8, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 9 to 368 of SEQ ID NO:8.

In a DNA wherein nucleotides of the sequence given in SEQ ID NO:4, or in a DNA wherein nucleotides of the sequence given in SEQ ID NO:8, are replaced by other nucleotides, such replacement is preferred when it does not alter the amino acid sequence of the complementarity determining regions (CDRs) coded for. This means that such replacement of nucleotides may occur in the inserts coding for the framework regions (FRs) or in a position where it does not alter the amino acid coded for due to the degeneracy of the triplet codons.

Likewise the invention relates to a recombinant DNA comprising an insert coding for a light chain murine variable domain of a monoclonal antibody selected from the group consisting of the antibodies FRP5, FSP16, FSP77 and FWP51, or coding for an amino acid sequence homologous to said light chain variable domain.

More specifically, the invention concerns a recombinant DNA comprising an insert coding for a light chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell lines FRP5, FSP16, FSP77 or FWP51, or which is homologous to genomic DNA of said cell lines and codes for an amino acid sequence homologous to the light chain variable domain of monoclonal antibodies FRP5, FSP16, FSP77 or FWP51. Particularly preferred is a recombinant DNA comprising an insert coding for a light chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell line FRP5, or which is homologous to genomic DNA of said cell line and codes for an amino acid sequence homologous to the light chain variable domain of monoclonal antibody FRP5, or a recombinant DNA comprising an insert coding for a light chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell line FwP51, or which is homologous to genomic DNA of said cell line and codes for an amino acid sequence homologous to the light chain variable domain of monoclonal antibody FWP51.

Preferred is a recombinant DNA comprising an insert coding for the polypeptide of formula II, wherein FR_5, FR_6, FR_7, FR_8, CDR_{1L}, CDR_{2L}, and CDR_{3L} have the meanings as mentioned hereinbefore, optionally further containing introns. Especially preferred is a recombinant DNA coding for the polypeptide of formula II comprising inserts coding for murine or human framework regions FR_5, FR_6, FR_7 and FR_8, and inserts coding for complementarity determining regions of the DNA sequence 480 to 512 (CDR_{1L}), the DNA sequence 558 to 578 (CDR_{2L}), and the DNA sequence 675 to 701 (CDR_{3L}) of SEQ ID NO:4, or coding for complementarity determining regions of the DNA sequence 483 to 515 (CDR_{1L}), the DNA sequence 561 to 581 (CDR_{2L}), and the DNA sequence 678 to 701 (CDR_{3L}) of SEQ ID NO:8.

Most preferred is a DNA comprising an insert of the DNA sequence 411 to 728 of SEQ ID NO:4, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 411 to 728 of SEQ ID NO:4. Likewise preferred is a DNA comprising an insert of the DNA sequence 414 to 728 of SEQ ID NO:8, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 414 to 728 of SEQ ID NO:8. In a DNA wherein nucleotides of the sequence given in SEQ ID NO:4, or in a DNA wherein nucleotides of the sequence given in SEQ ID NO:8, are replaced by other nucleotides, such replacement is preferred when it does not alter the amino acid sequence of the complementarity determining regions (CDRs) coded for, as is described above for DNA coding for the heavy chain variable domain.

For the assembly of complete tetrameric immunoglobulin molecules and the expression of chimeric antibodies, the recombinant DNA inserts coding for heavy and light chain variable domains are fused with the corresponding DNAs coding for heavy and light chain constant domains, then transferred into appropriate host

cells, for example after incorporation into hybrid vectors.

The invention therefore also concerns recombinant DNAs comprising an insert coding for a heavy chain murine variable domain of an antibody directed to the extracellular domain of c-erbB-2 fused to a human constant domain γ, for example γ1, γ2, γ3 or γ4, preferably γ1 or γ4. Likewise the invention concerns recombinant DNAs comprising an insert coding for a light chain murine variable domain of an antibody directed to the extracellular domain of c-erbB-2 fused to a human constant domain κ or λ, preferably κ.

The invention especially concerns recombinant DNAs coding for a single-chain recombinant antibody as defined hereinbefore, e.g. recombinant DNA wherein the heavy chain variable domain and the light chain variable domain are linked by way of a DNA insert coding for a spacer group, in particular a recombinant DNA coding for a protein of the formula III, wherein FR_1,FR_2,FR_3,FR_4,FR_5,FR_7,FR_8,FR_9, SP, CDR_{1H},CDR_{2H}, CDR_{3H}, CDR_{1L}, CDR_{2L} and CDR_{3L} have the meanings given above, optionally comprising further DNA coding for an effector molecule and/or signal sequences facilitating the processing of the antibody in the host cell. In particular the invention concerns a DNA comprising an insert of the DNA sequence 9-728 of SEQ ID NO:4, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, especially a DNA comprising an insert of the DNA sequence 9 to 728 of SEQ ID NO:4. Furthermore the invention relates to a DNA comprising an insert of the DNA sequence 9-728 of SEQ ID NO:8 wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, especially a DNA comprising an insert of the DNA sequence 9 to 728 of SEQ ID NO:8.

In another embodiment the invention pertains to recombinant DNAs coding for a recombinant DNA wherein the heavy chain variable domain and the light chain variable domain are linked by way of a DNA insert coding for a spacer group, optionally comprising a signal sequence facilitating the processing of the antibody in the host cell and/or a DNA coding for a peptide facilitating the purification of the antibody and/or a DNA coding for a cleavage site and/or a DNA coding for a peptide spacer and/or a DNA coding for an effector molecule.

The DNA coding for an effector molecule is intended to be a DNA coding for the above-mentioned effector molecules, particularly a DNA coding for alkaline phosphatase or Pseudomonas exotoxin A. The DNA encoding such an effector molecule has the sequence of a naturally occurring enzyme or toxin encoding DNA, or a mutant therof, and can be prepared by methods well known in the art. A mutant of the naturally occurring DNA encoding e.g. alkaline phosphatase or Pseudomonas exotoxin A, or a variant thereof can be obtained e.g. analogously to the methods described above.

Most preferred is a DNA comprising an insert of the DNA sequence 23 to 814 of SEQ ID NO:5, of the DNA sequence 86 to 2155 of SEQ ID NO:5 or of the DNA sequence 23 to 2155 of SEQ ID NO:5, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 23 to 2155 of SEQ ID NO:5.

Equally preferred is a DNA comprising an insert of the DNA sequence 1 to 1911 of SEQ ID NO: 10, of the DNA sequence 64 to 1911 of SEQ ID NO: 10, or of the DNA sequence 97 to 1911 of SEQ ID NO: 10, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 1 to 1911 of SEQ IDs NO: 10; or a DNA comprising an insert of the DNA sequence 1 to 1911 of SEQ ID NO: 11, of the DNA sequence 64 to 1911 of SEQ ID NO: 11, of the DNA sequence 96 to 1911 of SEQ ID NO: 11, or of the DNA sequence 97 to 1911 of SEQ ID NO: 11, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 1 to 1911 of SEQ ID NO: 11.

Furthermore the invention concerns a recombinant DNA which is a hybrid vector comprising an insert coding for the variable domain of a murine heavy chain as described hereinbefore and/or an insert coding for the variable domain of a murine light chain as described hereinbefore, an origin of replication or an autonomously replicating sequence, one or more dominant marker sequences and, optionally, expression control sequences, signal sequences and additional restriction sites.

In a first embodiment the hybrid vector according to the invention comprises an expression cassette comprising a promoter and a DNA coding for a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer, which DNA is controlled by said promoter, and isolating said protein.

In a second embodiment, the hybrid vector according to the invention comprises an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, and a fusion protein optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer.

Vectors typically perform two functions in collaboration with compatible host cells. One function is to facilitate the cloning of the nucleic acid that encodes the immunoglobulin variable domains, i.e. to produce usable quantities of the nucleic acid (cloning vectors). The other function is to provide for replication and expression of the recombinant gene constructs in a suitable host, either by maintenance as an extrachromosomal element or by integration into the host chromosome (expression vectors). A cloning vector comprises the recombinant gene constructs as described above, an origin of replication or an autonomously replicating sequence, dominant marker sequences and, optionally, signal sequences and additional restriction sites. An expression vector additionally comprises expression control sequences essential for the transcription and translation of the recombinant genes.

An origin of replication or an autonomously replicating sequence is provided either by construction of the vector to include an exogeneous origin such as derived from Simian virus 40 (SV 40) or another viral source, or by the host cell chromosomal mechanisms.

The markers allow for selection of host cells which contain the vector. Selection markers include genes which confer resistance to heavy metals such as copper or to antibiotics such as geneticin (G-418) or hygromycin, or genes which complement a genetic lesion of the host cell such as the absence of thymidin kinase, hypoxanthine phosphoryl transferase, dihydrofolate reductase or the like.

Signal sequences may be, for example, presequences or secretory leaders directing the secretion of the recombinant antibody, splice signals, or the like. Examples for signal sequences directing the secretion of the recombinant antibody are sequences derived from the ompA gene, the pelB (pectate lyase) gene or the phoA gene.

As expression control sequences, the vector DNA comprises a promoter, sequences necessary for the initiation and termination of transcription and for stabilizing the mRNA and, optionally, enhancers and further regulatory sequences.

A wide variety of promoting sequences may be employed, depending on the nature of the host cell. Promoters that are strong and at the same time well regulated are the most useful. Sequences for the initiation of translation are for example Shine-Dalgarno sequences. Sequences necessary for the initiation and termination of transcription and for stabilizing the mRNA are commonly available from the noncoding 5'-regions and 3'-regions, respectively, of viral or eukaryotic cDNAs, e.g. from the expression host. Enhancers are transcription-stimulating DNA sequences of viral origin, e.g. derived from Simian virus, polyoma virus, bovine papilloma virus or Moloney sarcoma virus, or of genomic, especially murine, origin.

The various DNA segments of the vector DNA are operationally linked, i.e. they are contiguous and placed into a functional relationship with each other.

Examples of vectors which are suitable for replication and expression in an E. coli strain are bacteriophages, for example derivatives of λ bacteriophages, or plasmids, such as, in particular, the plasmid ColE1 and its derivatives, for example pMB9, pSF2124, pBR317 or pBR322 and plasmids derived from pBR322, such as pUC9, pUCK0, pHRi148 and pLc24. Suitable vectors contain a complete replicon, a marker gene, recognition sequences for restriction endonucleases, so that the foreign DNA and, if appropriate, the expression control sequence can be inserted at these sites, and optionally signal sequences and enhancers.

Microbial promoters are, for example, the strong leftward promoter P_L of bacteriophage λ which is controlled by a temperature sensitive repressor. Also suitable are E. coli promoters such as the lac (lactose) promoter regulated by the lac repressor and induced by isopropyl-β-D-thiogalactoside, the trp (tryptophan) promoter regulated by the trp repressor and induced e.g. by tryptophan starvation, and the tac (hybrid trp-lac promoter) regulated by the lac repressor.

Vectors which are suitable for replication and expression in yeast contain a yeast replication start and a selective genetic marker for yeast. One group of such vectors includes so-called ars sequences (autonomous replication sequences) as origin of replication. These vectors are retained extrachromosomally within the yeast cell after the transformation and are replicated autonomously. Furthermore, vectors which contain all or part of the 2μ (2 mikron) plasmid DNA from Saccharomyces cerevisiae can be used. Such vectors will get integrated by recombination into 2μ plasmids already existing within the cell, or replicate autonomously. 2μ sequences are particularly suitable when high transformation frequency and high copy numbers are to be achieved.

Expression control sequences which are suitable for expression in yeast are, for example, those of highly expressed yeast genes. Thus, the promoters for the TRP1 gene, the ADHI or ADHII gene, acid phosphatase (PHO3 or PHO5) gene, isocytochrome gene or a promoter involved with the glycolytic pathway, such as the promoter of the enolase, glyceraldehyde-3-phosphate kinase (PGK), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase and glucokinase genes, can be used.

Vectors suitable for replication and expression in mammalian cells are preferably provided with promoting sequences derived from DNA of viral origin, e.g. from Simian virus 40 (SV40), Rous sarcoma virus (RSV),

adenovirus 2, bovine papilloma virus (BPV), pepovavirus BK mutant (BKV), or mouse or human cytomegalovirus (CMV). Alternatively, the vectors may comprise promoters from mammalian expression products, such as actin, collagen, myosin etc., or the native promoter and control sequences which are normally essociated with the desired gene sequence, i.e. the immunoglobulin H-chain or L-chain promoter.

Preferred vectors are suitable for both procaryotic and eucaryotic hosts and are based on viral replication systems. Particularly preferred are vectors comprising Simian virus promoters, e.g. pSVgpt or pSVneo, further comprising an enhancer, e.g. an enhancer normally associated with the immunoglobulin gene sequences, in particular the mouse Ig H- or L-chain enhancer.

The recombinant DNA coding for a recombinant entibody of the invention can be prepared, for example, by culturing a transformed host cell and optionally isolating the prepared DNA.

In particuler, such DNA can be prepered by a method comprising

a) preparing murine DNA coding for the veriable heavy and/or light chain domains of the entibody with the desired specificity, e.g. by isolating the DNA from the genome of a suitable hybridoma cell line and selecting the desired DNA using DNA probes, or by isolating mRNA from e suitable hybridoma cell line and prepering cDNA coding for the variable heavy and/or light chain domains of the entibody with the desired specificity using oligonucleotide primers,

b) preparing DNA coding for the desired signal sequence and/or preparing DNA coding for an effector molecule, e.g. by isoleting the desired DNA(s) from a suitable source, e.g. from a genomic library or a cDNA library using DNA probes,

c) synthesizing DNA coding for the desired specer group by chemical methods,

d) constructing recombinant genes encoding the recombinent entibodies by incorporating the DNA of step a) and, optionally, b) and/or c) into appropriate hybrid vectors,

e) transferring the obtained hybrid vectors into a recipient host cell or retrieving the DNA coding for the recombinant genes and transferring the unlinked DNA into a reciplent host cell,

f) selecting and culturing the transformed host cell, and

g) optionally isolating the desired DNA.

The DNA according to step a) of the process described above can be obtained by isoletion of genomic DNA or by preparation of cDNA from isolated mRNA. Genomic DNA from hybridoma cells is isolated by methods known in the art which include steps for disruption of the cells, e.g. by lysis in presence of detergents like Triton™, extracting the DNA, e.g. by treatment with phenol and $CHCl_3$/isoamyl alcohol, and precipitation of DNA. The DNA is fragmented, conveniently by one or more restriction endonucleases, the resulting fragments are replicated on a suitable carrier, e.g. nitrocellulose membranes, and screened with a DNA probe for the presence of the DNA sequences coding for the polypeptide sequence of interest, in particular for the presence of the rearranged H- and L-chain Ig gene loci. By this procedure DNA fragments are found that contain inserts with heavy chein V, D and J regions and light chain V and J regions, respectively, together with a leader sequence and introns, if any. cDNA from hybridoma cells is likewise prepared by methods known in the art, e.g. by extracting total cellular RNA, isolating mRNA by a suitable chromatographic method, e.g. chromatography on oligo(dT)-cellulose, synthesizing cDNA with a mixture of deoxynucleotide triphosphates and reverse transcriptase in the presence of oligonucleotide primers complementary to suitable regions in the murine immunoglobulin heevy and light chain constant domain genes, end isolating the cDNA. As a tool simplifying DNA isolation, the desired genomic DNA or cDNA may be amplified using polymerase chain reaction (PCR) technology. PCR involves repeated rounds of extension from two primers specific for DNA regions at each end of the gene. Preferably, cDNA transcripts of total mRNA from the suitable hybridoma cell line is treated in a heating/cooling cycle with Taq DNA polymerase in the presence of primers tailored to hybridize to Ig H- end L-chein variable domains, respectively.

Genomic DNA or cDNA according to step b) of the process described above is isolated from suitable becterial or mammalian cells according to methods known in the art. Preferably, the methods as described under a) are used, substituting the corresponding source cells for the murine hybridome cells and using DNA probes designed to hybridize with the desired signal sequences or the genes coding for the desired effector molecules. In bacteria wherein separation of mRNA from total RNA is not possible with olig(dT)-cellulose, cDNA is prepared from total RNA using corresponding oligonucleotide primers. The DNA isolation is simplified considerably by the PCR technology.

DNA according to step c) is prepared by conventional chemical and enzymatic methods, e.g. by chemical synthesis of oligonucleotides of between thirty end sixty bases with overlepping complementary sequences, hybridization of such oligonucleotides, and enzymatic ligation, optionally after filling-in of missing beses with suitable enzymes in the presence of the corresponding deoxynucleotide triphosphates.

The DNA probe for the mouse veriable chain domains may be a synthetic DNA, e cDNA derived from mRNA coding for the desired immunoglobulin or a genomic DNA or DNA fragment of known nucleotide sequence. As

probes for the detection and/or amplification of the rearranged Ig gene loci of the variable domains of L-/H-chains, DNA fragments of known nucleotide sequences of adjacent conserved variable or constant domains are selected which constitute the Ig loci of the L-/H-chain in the mammal from which the DNA is derived, e.g. Balb/c mice. The DNA probe is synthesized by chemical methods or isolated from suitable tissue of an appropriate mammal, e.g. Balb/c mouse liver, and purified by standard methods. If required, the probe DNA is labelled, e.g. radioactively labelled by the well-known nick-translation technique, then hybridized with the DNA library in buffer and salt solutions containing adjuncts, e.g. calcium chelators, viscosity regulating compounds, proteins, non-specific DNA and the like, at temperatures favoring selective hybridization.

Once a fragment has been identified which contains the desired DNA sequence, this fragment may be further manipulated to remove nonessential DNA, modified at one or both termini, and treated to remove all or a portion of intervening sequences, or the like.

The joining of the various DNA fragments in order to produce recombinant genes encoding the recombinant antibodies is performed in accordance with conventional techniques, for example, by blunt- or staggered-end ligation, restriction enzyme digestion to provide for appropriate cohesive termini, filling-in cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases.

The transfer of the recombinant DNAs, e.g. the transfer of hybrid vectors, and the selection of transformed cells is described below.

Moreover, the invention relates to host cells transformed with the recombinant DNAs described above, namely host cells which are transformed with a DNA encoding the heavy chain and/or a DNA encoding the light chain of the desired recombinant antibody, in particular host cells transformed with a DNA encoding the preferred single-chain recombinant antibody.

More specifically, the invention concerns a host cell which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter and a DNA coding for a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein further comprising a peptide facilitating purification, a cleavage site and a peptide spacer which DNA is controlled by said promoter.

Furthermore, the invention pertains to a host cell which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein further comprising a peptide facilitating purification, a cleavage site and a peptide spacer.

In particular, the present invention relates to a process for the production of a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein further comprising a peptide facilitating purification, a cleavage site and a peptide spacer comprising culturing a host, e.g. E. coli, which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding said protein, and isolating said protein.

The host cells of the present invention have to be capable of culture in vitro. Suitable host cells are of procaryotic or of eucaryotic origin and are, for example, bacterial cells, e.g. E. coli, yeasts, e.g. Saccharomyces cerevisiae, or mammalian cells. For the preparation of functional chimeric human/mouse antibodies the host cells have to be of higher eucaryotic origin to provide a suitable environment for the production of active antibodies, since the biosynthesis of functional tetrameric antibody molecules requires correct nascent polypeptide chain folding, glycosylation, and assembly.

Examples of suitable hosts are microorganisms which are devoid of or poor in restriction enzymes or modification enzymes, such as bacteria, in particular strains of Escherichia coli, for example E. coli X1776, E. coli Y1090, E. coli HB 101, E. coli W3110, E. coli HB101/LM1035, E. coli JA 221, E. coli DH5α, E. coli K12, or E. coli CC118 strain, Bacillus subtilis, Bacillus stearothermophilus, Pseudomonas, Haemophilus, Streptococcus and others, and yeasts, for example Saccharomyces cerevisiae such as S. cerevisiae GRF 18. Further suitable host cells are cells of higher organisms, in particular established continuous human or animal cell lines, e.g. human embryonic lung fibroblasts L132, human malignant melanoma Bowes cells, HeLa cells, SV40 virus transformed kidney cells of African green monkey COS-7 or Chinese hamster ovary (CHO) cells, or cells of lymphoid origin, such as lymphoma, myeloma, hybridoma, trioma or quadroma cells, for example PAI, Sp2/0 or X63-Ag8.653 cells.

The above mentioned strains of E. coli, in particular E. coli CC118, are preferred as hosts.

The invention also concerns processes for the preparation of transformed host cells wherein suitable recipient host cells as described hereinbefore are transformed with a hybrid vector according to the invention, and the transformed cells are selected.

Transformation of microorganisms is carried out as described in the literature, for example for S. cerevisiae (A.Hinnen et al., Proc. Natl. Acad. Sci. USA 75: 1929, 1978), for B. subtilis (Anagnostopoulos et al., J. Bacteriol. 81: 741, 1961), and for E. coli (M. Mandel et al., J. Mol. Biol. 53: 159, 1970).

Accordingly, the transformation procedure of E. coli cells includes, for example, Ca^{2+} pretreatment of the cells so as to allow DNA uptake, and incubation with the hybrid vector. The subsequent selection of the transformed cells can be achieved, for example, by transferring the cells to a selective growth medium which allows separation of the transformed cells from the parent cells dependent on the nature of the marker sequence of the vector DNA. Preferably, a growth medium is used which does not allow growth of cells which do not contain the vector. The transformation of yeast comprises, for example, steps of enzymatic removal of the yeast cell wall by means of glucosidases, treatment of the obtained spheroplasts with the vector in the presence of polyethylene glycol and Ca^{2+} ions, and regeneration of the cell wall by embedding the spheroplasts into agar. Preferably, the regeneration agar is prepared in a way to allow regeneration and selection of the transformed cells as described above at the same time.

Transformation of cells of higher eucaryotic origin, such as mammalian cell lines, is preferably achieved by transfection. Transfection is carried out by conventional techniques, such as calcium phosphate precipitation, microinjection, protoplast fusion, electroporation, i.e. introduction of DNA by a short electrical pulse which transiently increases the permeability of the cell membrane, or in the presence of helper compounds such as diethylaminoethyldextran, dimethyl sulfoxide, glycerol or polyethylene glycol, and the like. After the transfection procedure, transfected cells are identified and selected, for example, by cultivation in a selective medium chosen depending on the nature of the selection marker, for example standard culture media such as Dulbecco's modified Eagle medium (DMEM), minimum essential medium, RPMI 1640 medium and the like, containing e.g. the corresponding antibiotic.

The host cells are transformed with the recombinant L-chain gene construct alone, with the recombinant H-chain gene construct alone, with both, either sequentially or simultaneously, or by using a vector construct comprising both the L-chain and H-chain genes, for example a recombinant single-chain antibody gene construct as indicated hereinbefore.

Preferred are host cells transformed with a recombinant single-chain antibody gene construct comprising DNA coding for the heavy chain variable domain of an anti-c-erbB-2 antibody, DNA coding for a spacer group, DNA coding for the light chain variable domain of an anti-c-erbB-2 antibody and DNA coding for an effector molecule, in particular transfected with the preferred recombinant single-chain antibody gene construct as indicated hereinbefore. Further examples of host cells of the invention are cells transfected with similar recombinant plasmids which contain alternative orientations of the H- and L-chain gene constructs, and those incorporating additional DNA elements to facilitate high levels of expression of the recombinant antibodies.

The host cells of the invention are genetically stable, secrete recombinant antibodies of the invention of constant specificity and can be activated from deep-frozen cultures by thawing and recloning.

The transformed host cells are cultured by methods known in the art in a liquid medium containing assimilable sources of carbon, e.g. carbohydrates such as glucose or lactose, nitrogen, e.g. amino acids, peptides, proteins or their degradation products such as peptones, ammonium salts or the like, and inorganic salts, e.g. sulfates, phosphates and/or carbonates of sodium, potassium, magnesium and calcium. The medium furthermore contains, for example, growth-promoting substances, such as trace elements, for example iron, zinc, manganese and the like.

The medium is preferably so chosen as to exert a selection pressure and prevent the growth of cells which have not been transformed or have lost the hybrid vector. Thus, for example, an antibiotic is added to the medium if the hybrid vector contains an antibiotic resistance gene as marker. If, for instance, a host cell is used which is auxotrophic in an essential amino acid whereas the hybrid vector contains a gene coding for an enzyme which complements the host defect, a minimal medium deficient of said amino acid is used to culture the transformed cells.

Cells of higher eucaryotic origin such as mammalian cells are grown under tissue culture conditions using commercially available media, for example Dulbecco's modified Eagle medium (DMEM), minimum essential medium, RPMI 1640 medium and the like as mentioned above, optionally supplemented with growth-promoting substances and/or mammalian sera. Techniques for cell cultivation under tissue culture condition are well known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapped cell culture, e.g. in hollow fibres, microcapsules, on agarose microbeads, porous glass beads, ceramic cartridges, or other microcarriers.

Culturing is effected by processes which are known in the art. The culture conditions, such as temperature, pH value of the medium and fermentation time, are chosen so that a maximum titer of the polypeptide or derivative of the invention is obtained. Thus, an E. coli or yeast strain is preferably cultured under aerobic conditions by submerged culture with shaking or stirring at a temperature of about 20°C to 40°C, preferably at about 30°C, and a pH value of 4 to 8, preferably of about pH 7, for about 4 to 30 hours, preferably until maximum yields of the polypeptide or derivative of the invention are reached.

When the cell density has reached a sufficient value, the culture is interrupted and the polypeptide or derivative can be isolated. If the hybrid vector contains a suitable secretion signal sequence, the polypeptide or derivative is secreted by the transformed cell directly into the culture medium. Otherwise, the cells have to be destroyed, for example by treatment with a detergent such as SDS, NP-40™, Triton™ or deoxycholic acid, lysed with lysozyme or a similarly acting enzyme, or disrupted by an osmotic shock or ultra-sound. Break-up of the cells will also be required if the signal sequence directs the secretion of the desired protein into the cell periplasm. If yeast is used as a host microorganism, the cell wall may be removed by enzymatic digestion with a glucosidase. Alternatively or additionally, mechanical forces, such as shearing forces (e.g. French press, Dyno mill and the like) or shaking with glass beads or aluminium oxide, or alternating freezing, for example in liquid nitrogen, and thawing, for example at 30°C to 40°C, as well as ultra-sound can be used to break the cells.

The cell supernatant or the solution obtained after centrifugation of the mixture obtained after breaking the cells, which contains proteins, nucleic acids and other cell constituents, is enriched in proteins, including the polypeptides of the invention, in a manner which is known per se. Thus, for example, most of the non-protein constituents are removed by polyethyleneimine treatment and the proteins including the polypeptides and derivatives of the invention are precipitated, for example, by saturation of the solution with ammonium sulfate or with other salts. Otherwise, the cell supernatant or lysate is directly pre-purified by filtering through suitable membranes and/or with chromatographic methods, for example affinity chromatography.

The recombinant antibodies and the monoclonal antibodies according to the invention can be used for the qualitative and quantitative determination of the extracellular domain of the growth factor receptor c-erbB-2. This is especially useful for the monitoring of tumor progression, for the decision whether a tumor is amenable to treatment with the recombinant or monoclonal antibodies of the invention, and for monitoring the treatment of tumor with chemotherapy. Tumors considered are those over-expressing c-erbB-2, for example breast and ovarian tumors.

In general, the monoclonal and the recombinant antibodies according to the invention can be used in any of the known immunoassays which rely on the binding interaction between the antibodies and the antigen, i.e. the extracellular domain of the c-erbB-2 protein. Examples of such assays are radio-, enzyme, fluorescence, chemiluminescence, immunoprecipitation, latex agglutination, and hemagglutination immunoassays, and, in particular, immunostaining methods.

The antibodies according to the invention can be used as such or in the form of enzyme-conjugated derivatives in an enzyme immunoassay. Any of the known modifications of an enzyme immunoassay can be used, for example soluble phase (homogeneous) enzyme immunoassay, solid phase (heterogeneous) enzyme immunoassay, single enzyme immunoassay or double (sandwich) enzyme immunoassay with direct or indirect (competitive) determination of the c-erbB-2 protein.

An example of such an enzyme immunoassay is a sandwich enzyme immunoassay in which a suitable carrier, for example the plastic surface of a microtiter plate or of a test tube, e.g. of polystyrene, polypropylene or polyvinylchloride, glass or plastic beads, filter paper, dextran etc. cellulose acetate or nitrocellulose sheets, magnetic particles or the like, is coated with a monoclonal antibody of the invention by simple adsorption or optionally after activation of the carrier, for example with glutaraldehyde or cyanogen bromide. Then test solutions containing the soluble c-erbB-2 protein and finally single-chain recombinant antibodies of the invention comprising a detectable enzyme, e.g. alkaline phosphatase, are added. The amount of the soluble c-erbB-2 protein in the test solution is directly proportional to the amount of bound recombinant antibody and is determined by adding an enzyme substrate solution. The enzyme substrate reaction results, for example, in a color change which can be observed by eye or with optical measuring devices.

The antibodies according to the invention can be used as such or in the form of radioactively labelled derivatives in a radioimmunoassay (RIA). As described above for enzyme immunoassays, any of the known modifications of a radioimmunoassay can be used.

The tests are carried out in an analogous manner to the enzyme immunoassays described above using a radioactive label, e.g. ^{125}I, instead of an enzyme label. The amount of immune complex formed which corresponds to the amount of c-erbB-2 protein present in the test solutions is determined by measuring the radioactivity of the immune complex.

For immunostaining cryosections of cryopreserved biopsy material or paraffin embedded tissue sections are treated with a solution containing a recombinant antibody of the invention comprising a detectable enzyme.

Bound recombinant antibody is detected by treatment with a suitable enzyme substrate, preferably an enzyme substrate which leads to a solid deposit (stain) at the site of the recombinant antibody of the invention. In place of recombinant antibodies comprising an enzyme, a recombinant antibody comprising streptavidin and a solution of a biotin-enzyme-conjugate may be used, which leads to higher enzyme concentration at the site of the antibody and hence increased sensitivity of the immunostaining method. The solid deposit of the enzyme substrate is detected by inspection with a microscope, for example with a fluorescence microscope, or by scanning the optical density at the wavelength of the stain.

The use according to the invention of recombinant and/or monoclonal antibodies as described hereinbefore for the determination of c-erbB-2 protein also includes other immunoassays known per se, for example immunofluorescence assays, latex agglutination with antibody-coated or antigen coated latex particles, hemagglutination with antibody-coated or antigen-coated red blood corpuscles, evanescent light assays using an antibody-coated optical fibre and other direct-acting immunosensors which convert the binding event into an electrical or optical signal, or the like.

The invention also concerns test kits for the qualitative and quantitative determination of c-erbB-2 protein comprising recombinant antibodies of the invention and/or monoclonal antibodies of the invention and, optionally, adjuncts.

Test kits according to the invention for an enzyme immunoassay contain, for example, a suitable carrier, optionally freeze-dried solutions of a monoclonal antibody, optionally freeze-dried or concentrated solutions of a recombinant antibody comprising an enzyme or streptavidin, solutions of an enzyme-biotin conjugate if a recombinant antibody comprising streptavidin is used, enzyme substrate in solid or dissolved form, standard solutions of c-erbB-2 protein, buffer solutions, and, optionally, polypeptides or detergents for preventing non-specific adsorption and aggregate formation, pipettes, reaction vessels, calibration curves, instruction manuals and the like.

Test kits according to the invention for immunostaining contain, for example, optionally freeze-dried or concentrated solutions of a recombinant antibody comprising an enzyme or streptavidin, solutions of an enzyme-biotin conjugate if a recombinant antibody comprising streptavidin is used, enzyme substrate in solid or dissolved form, buffer solutions, and, optionally, pipettes, reaction vessels, calibration curves, instruction manuals and the like.

The recombinant and monoclonal antibodies of the invention can be used for the qualitative and quantitative determination of c-erbB-2 protein. Due to the fact that the growth factor receptor c-erbB-2 is overexpressed in certain tumor types, for example breast and ovarian tumors, the antibodies are particularly well suited for detection and monitoring of the mentioned tumors. In addition, radiolabelled derivatives of the antibodies of the invention may be used for the in vivo localization of tumors in a patient using radioscanning techniques. To that end, radiolabelled derivatives of antibodies of the invention are injected into the patient, and the patient scanned with a gamma imager at regular intervals. Cells over-expressing the growth factor receptor c-erbB-2 will take up more radioactive antibodies than other tissue and will be clearly recognized by the gamma imaging camera. Preferentially recombinant or monoclonal antibodies labelled with ^{131}I or with ^{99m}Tc are used for radioscanning in amounts of 3 to 8 µg representing 15 to 30 µCi per kg body weight.

The antibodies of the invention can further be used for the isolation and purification of the c-erbB-2 protein from natural sources or from transformed host cells by immunoaffinity chromatography.

Furthermore, the monoclonal antibodies and the recombinant antibodies of the invention, in particular recombinant antibodies comprising an effector molecule, especially a toxin, in particular Pseudomonas exotoxin, are useful for the treatment of patients with tumors over-expressing the growth factor receptor c-erbB-2, for example breast or ovarian tumors. If it is desired, tumor therapy may comprise applying more than one, e.g. two different, antibodies of the invention, for example applying both FRP5 and FWP51. The recombinant antibodies comprising a phosphatase may be used in connection with a phosphorylated prodrug such as mitomycin phosphate or etoposide phosphate, thus enabling the conversion of the active drug to the prodrug at the site of the tumor.

The invention therefore also concerns pharmaceutical compositions for treating tumors over-expressing the growth factor receptor c-erbB-2 comprising a therapeutically effective amount of a recombinant antibody or of a monoclonal antibody according to the invention and a pharmaceutically acceptable carrier. Preferred are pharmaceutical compositions for parenteral application. Compositions for intramuscular, subcutaneous or intravenous application are e.g. isotonic aqueous solutions or suspensions, optionally prepared shortly before use from lyophilized or concentrated preparations. Suspensions in oil contain as oily component the vegetable, synthetic or semi-synthetic oils customary for injection purposes. The pharmaceutical compositions may be sterilized and contain adjuncts, e.g. for conserving, stabilizing, wetting, emulsifying or solubilizing the ingredients, salts for the regulation of the osmotic pressure, buffer and/or compounds regulating the viscosity, e.g. sodium carboxycellulose, carboxymethylcellulose, sodium carboxymethylcellulose, dextran, polyvinylpyrroll-

dine or gelatine.

The pharmaceutical compositions of the invention contain from approximately 0.01% to approximately 50% of active ingredients. They may be in dosage unit form, such as ready-to-use ampoules or vials, or also in lyophylized solid form.

In general, the therapeutically effective dose for mammals is between approximately 5 and 25 µg of a recombinant antibody of the invention or of a monoclonal antibody of the invention per kg body weight depending on the type of antibody, the status of the patient and the mode of application. The specific mode of administration and the appropriate dosage will be selected by the attending physician taking into account the particulars of the patient, the state of the disease, the type of tumor treated, and the like. The pharmaceutical compositions of the invention are prepared by methods known in the art, e.g. by conventional mixing, dissolving, confectioning or lyophilizing processes. Pharmaceutical compositions for injection are processed, filled into ampoules or vials, and sealed under aseptic conditions according to methods known in the art.

The invention particularly concerns the monoclonal antibodies, the hybridoma cell lines, the recombinant single-chain antibodies, the recombinant DNAs, the transformed host cells, and the methods for the preparation thereof as described in the Examples. The following examples illustrate the invention but do not limit it to any extent.

Abbreviations

ATP	adenosine triphosphate
BSS	Earle's balanced salt solution
BSA	bovine serum albumin
DEAE	diethylaminoethyl
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	disodium ethylenediaminetetraacetate
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
FCS	fetal calf serum
HAT medium	hypoxanthine, aminopterin and thymidine medium
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HT medium	hypoxanthine and thymidine medium
Ig	immunoglobulin
IPTG	isopropyl-β-thiogalactoside
MAb	monoclonal antibody
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tris	Tris-(hydroxymethyl)-aminomethane
U	unit
V_L	light chain variable domain
V_H	heavy chain variable domain
XP	5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt

Examples

Example 1. Preparation of hybridoma cell lines FRP5, FSP16, FWP51 and FSP77

1.1 Source of antigen and immunization of Balb/c mice: The SKBR3 human breast tumor cell line (ATCC HTB 30), isolated in 1970 from a pleural effusion of a breast cancer patient, expresses approximately 1 x 10^6 molecules of the c-erbB-2 receptor protein per cell. 20 x 10^6 SKBR3 cells in PBS are injected subcutaneously and/or intra-peritoneally into Balb/c mice. The cells are mixed 1:1 (v/v) with complete Freund's adjuvant. The injections are repeated a total of five times over the period of approximately 3 months replacing Freund's incomplete adjuvant for complete adjuvant. The final injection of cells is given three days before the fusion.

1.2 Cell fusion: Immunized mice are sacrificed and their splenocytes fused according to conventional methods (Koehler & Milstein, Nature 256:495, 1976). Spleen cells are mixed at a 5:1 to 10:1 ratio with the fusion

partner, the mouse myeloma cell line PAI (Stoker et al., Research Disclosure #21713, 1982), in the presence of 41 % polyethylene glycol 4000 (Merck). Fused cells are plated at a density of 1 x 10^6 cells per well in 24-well microtiter plates on peritoneal macrophages and fed 3 times per week with standard HAT selection medium for 2 weeks followed by 2 weeks of HT medium. When the growth of hybridoma cells becomes visible, the supernatants are screened as described in Example 1.3. Positive hybridomas are cloned and stored.

1.3 Antibody detection in hybridoma supernatants: Culture fluids of growing hybridomas are tested for the presence of anti-c-erbB-2 antibody using a protocol involving two steps, immunofluorescence and immunoprecipitation.

1.3.1 Immunofluorescence: In the first step, hybridoma supernatants are tested for their immunofluorescent staining of mouse cells expressing high levels of the human c-erbB-2 protein. To isolate these cells the HC11 mouse mammary epithelial cell line (Ball et al., EMBO J. 7: 2089, 1988) is transfected according to conventional, previously described methods (Graham & van der Eb, Virology 52: 456, 1973) with a plasmid expressing the human c-erbB-2 protein (Masuko et al., Jpn. Cancer Res. 80: 10, 1989) and with the plasmid pSV2neo (Southern & Berg, J. Mol. Appl. Genet. 1: 327, 1982) which encodes the gene for resistance to the drug G418. Transfected cells are selected 2 weeks in medium containing 200 µg/ml G418 (Geneticin, Gibco-BRL). Individual clones are selected and analyzed for expression of the human c-erbB-2 protein using conventional protein blotting techniques (Towbin et al., Proc. Natl. Acad. Sci. USA 76: 4350, 1979). A clone expressing high levels of the human c-erbB-2 protein (clone R1#11) is selected and used in the immunofluorescent assay. Non-transfected HC11 cells serve as control cells.

The assay is done in the following manner: The cells (R1 #11 or HC11) are grown in RPMI medium containing 8 % heat inactivated FCS (Amimed), 10 ng/ml EGF (Inotech) and 5 µg/ml insulin (Sigma) for 1 -2 days on fibronectin (Boehringer Mannheim) coated cover slips. Fibronectin coated cover slips are prepared and stored at room temperature and they are used routinely for screening. The coverslips are rinsed in PBS containing calcium and magnesium and fixed by treatment for 10 min with 3.7 % formaldehyde (v/v in PBS). To reduce the non-specific binding the coverslips are incubated 20 min in PBS containing 3 % BSA (Sigma). The coverslips are washed in PBS and in water, then allowed to dry at room temperature. 20 - 30 µl of hybridoma supernatants are added to circled areas on a coverslip which is incubated 1 - 2 h at room temperature in a humified atmosphere. The coverslips are then washed three times with PBS containing 0.05 % Triton-X100™ (Fluka) and incubated an additional hour with anti-mouse Ig, fluorescein-linked whole antibody from sheep (Amersham). After three washes with PBS and one wash with water the cells are screened for fluorescence using a fluorescence microscope and a water immersion lens. Those hybridoma supernatants which are positive are screened in the second step described in Example 1.3.2.

1.3.2 Immunoprecipitation and protein blotting analysis: The SKBR3 human breast tumor cells express approximately 1 x 10^6 molecules of the c-erbB-2 protein per cell. A cell lysate is prepared by extracting approximately 4 x 10^6 cells in 1 ml of buffer containing 1 % Triton-X100™ (Fluka), 50 mM Tris-HCl, pH 7.5, 5 mM EGTA, 0.15 M NaCl, 1 mM PMSF (Boehringer Mannheim), 80 µg/ml aprotinin (Boehringer Mannheim), 50 µg/ml leupeptin (Boehringer Mannheim), and 4 µg/ml pepstatin (Boehringer Mannheim). 200 -500 µl supernatant of hybridomas which are positive in the immunofluorescence assay described in Example 1.3.1 are incubated with 100 µl of the SKBR3 extract (2.5 - 4.0 mg/ml). This amount of extract contains approximately 50 - 100 ng of c-erbB-2 protein. The hybridoma supernatants and SKBR3 extract are incubated overnight on ice, then 1 µl of the IgG fraction of sheep anti-mouse Ig (ICN Immunobiologicals) is added. The complexes are collected by the addition of Protein-A Sepharose™ (Pharmacia), washed with TNET (140 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 % Triton X-100™) and water, boiled in sample buffer (80 mM Tris-HCl, pH 6.8, 0.2 % SDS, 10 % glycerol) and the supernatants loaded onto 8 % SDS-PAGE. The proteins are electrophoresed and blotted onto PVDF membranes (Millipore) using a technique originally described by Towbin et al. (Proc. Natl. Acad. Sci. USA 76: 4350, 1979) with some modifications. The proteins are transferred using a semi-dry blotter (G. Frobel, Model 1004.01) following the instructions of the manufacturer. The membranes are blocked in PBS containing 0.5 % gelatin (Merck) for 1 h at 37°C. The membranes are washed twice for 5 min at 37°C in PTG (PBS containing 0.02 % gelatin (Merck) and 0.25 % Triton-X100™ (Fluka)). The c-erbB-2 protein is detected by incubating the membrane 45 min at 37°C in PTG containing an antiserum which is raised against the carboxy terminal 13 amino acids of the c-erbB-2 protein (Gullick et al., Int. J. Cancer 40: 246, 1987, antiserum 21N). The membranes are washed 3 times for 5 min at 37°C in PTG. The membrane-bound 21N antiserum is detected by incubating the membrane in PTG containing 0.1 µC/ml ^{125}I-labeled protein-A (Amersham). The membranes are washed 4 times for 5 min at 37°C in PTG and exposed to X-ray film. The hybridomas whose supernatants are able to specifically immunoprecipitate the c-erbB-2 protein are grown for single cell cloning and further characterization described below.

Example 2. Characterization of c-erbB-2 specific MAbs

2.1 Hybridoma storage and processing: Hybridoma FRP5, FSP16, FWP51 and FSP77 secreting anti-c-erbB-2 MAb FRP5, FSP16, FWP51 and FSP77, respectively, can be grown in culture, frozen at -80°C or in liquid nitrogen and recultivated. The cells are cloned by the method of limiting dilution and have been deposited with the European Collection of Animal Cell Lines in England. The hybridoma cell lines have the following access numbers: FRP5: 90112115, FSP16: 90112116, FSP77: 90112117, FWP51: 90112118. The cells are expanded by forming ascites in Balb/c mice primed with pristane. The antibodies are purified from the ascites by ammonium sulfate precipitation and ion exchange chromatography on DE 52 DEAE-cellulose columns (Whatman). Purified MAbs are stored in PBS at -80°C.

2.2 Isotyping of the MAbs: The isotype of the MAbs FRP5, FSP16, FWP51 and FSP77 is determined by ELISA analysis with rabbit antisera to mouse Ig classes and sub-classes (Biorad Mouse Typer TMSub Isotyping Kit™) as per manufacturer's suggested procedure. MAbs FRP5, FWP51, and FSP77 are of the IgG1 isotype, while FSP16 is of the IgG2b isotype. The light chains of all the MAbs are of the kappa type.

2.3 Flow cytometry: A FACS analysis using the c-erbB-2 specific MAbs is carried out as follows: SKBR3 human breast tumor cells are trypsinized, washed in FACS medium (BSS containing 10 µM sodium azide, 4 % FCS and 25 mM EDTA), and 1 x 10^6 cells are resuspended in 100 µl of FACS medium. Non-specific binding sites are blocked by incubating the cells 10 min at room temperature with 5 µl of goat serum. The SKBR3 cells are collected by centrifugation, resuspended in 50 µl of a 1:2 dilution of the supernatant made in FACS medium and incubated 45 min on ice. The cells are washed with 4 ml FACS medium, collected by centrifugation, resuspended in 50 µl of FACS medium containing a 1:20 dilution of anti-mouse Ig, fluorescein-linked whole antibody from sheep (Amersham), and incubated for 30 min on ice. 4 ml of FACS medium are added, the cells are collected by centrifugation, resuspended in 100 µl of FACS medium and analyzed without fixation for their fluorescence in a Becton-Dickinson FACScan™. As a control, SKBR3 cells are incubated with a non-reacting IgG1 MAb (1236S31-3). The FACS analysis shows that the SKBR3 cells treated with MAb FRP5, FSP16, FWP51, and FSP77 have a higher fluorescence than cells treated with the control MAb. These results show that the MAbs bind to the extracellular domain of the c-erbB-2 protein.

2.4 Binding domain of c-erbB-2 specific MAbs: MAbs FRP5 and FSP77 are covalently linked with ^{125}I (as carrier free sodium ^{125}iodide, Amersham) to a specific activity of 1 µC/µg using Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril, Sigma) according to a standard protocol (Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988, p. 330). Competition experiments are conducted by incubating SKBR3 cells (0.5 - 1 x 10^5 cells per 15 mm well, Nunclon™ 4-well multidish) with 250 µl RIA buffer (120 mM NaCl, 50 mM HEPES, pH 7.8, 1 mM EDTA, 2 % BSA) containing labeled FRP5 or FSP77 and varying amounts of unlabeled MAb FRP5, FSP16, FWP51 and FSP77 for 2 h at 4°C. The cells are washed 5 times with the RIA buffer, solubilized in 0.5 ml 1 % Triton X-100™, 10 % glycerol, 20 mM HEPES, pH 7.4, for 30 min at room temperature and the bound radioactivity is measured in a gamma counter. The results show that MAbs FRP5 and FSP16 compete with each other for binding to SKBR3 cells which suggests that these 2 MAbs bind to the same domain on the c-erbB-2 protein. MAbs FWP51 and FSP77 neither compete with each other nor with FRP5 or FSP16 for binding to the c-erbB-2 protein. In conclusion, the panel of 4 MAbs bind to 3 different domains of the extracellular portion of the c-erbB-2 membrane receptor tyrosine kinase.

Example 3. Isolation of RNA from the hybridoma cell line FRP5

3.1 Growth of FRP5 cells: FRP5 hybridoma cells (1 x 10^8) are grown in suspension culture at 37°C in DMEM (Seromed) further containing 10 % FCS (Amimed), 1 mM sodium pyruvate (Seromed), 2 mM glutamine (Seromed), 50 µM 2-mercaptoethanol and 100 µg/ml of gentamycin (Seromed) in a humidified atmosphere of air and 7.5 % CO_2 in 175 cm tissue culture flasks (Falcon 3028). The cells are harvested by centrifugation, washed once in PBS, flash frozen in liquid nitrogen and kept frozen as a pellet at - 80°C in a clean, sterile plastic capped tube.

3.2 Extraction of total cellular RNA from FRP5 cells: Total RNA is extracted using the acid guanidinium thiocyanate-phenol-chloroform method described by Chomczynski & Sacchi (Anal. Biochem. 162: 156, 1987). Cell pellets of FRP5 cells (1 x 10^8) are thawed directly in the tube in the presence of 10 ml of denaturing solution (4 M guanidinium thiocyanate (Fluka), 25 mM sodium citrate, pH 7.0, 0.5 % N-lauroylsarcosine (Sigma), 0.1 M 2-mercaptoethanol). The solution is homogenized at room temperature. Sequentially, 1 ml of 2 M sodium acetate, pH 4, 10 ml of phenol (water saturated) and 2 ml of chloroform-isoamyl alcohol mixture (49:1) are added to the homogenate. The final suspension is shaken vigorously for 10 sec and cooled on ice for 15 min. The samples are centrifuged at 10,000 x g for 20 min at 4°C. After centrifugation, RNA which is present in the aqueous phase is mixed with 10 ml of isopropanol and placed at -20°C for 1 h. The RNA precipitate is collected

by centrifugation, the pellet dissolved in 3 ml water and the RNA reprecipitated by addition of 1 volume of isopropanol at -20°C. After centrifugation and washing the pellet in ethanol, the final pellet of RNA is dissolved in water. The method yields approximately 300 µg of total cellular RNA. The final purified material is stored frozen at -20°C.

3.3 Isolation of poly(A) containing RNA: Poly(A) containing RNA is selected from total RNA by chromatography on oligo(dT)-cellulose (Boehringer Mannheim) as described originally by Edmonds et al. (Proc. Natl. Acad. Sci. USA 68: 1336, 1971) and modified by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982, p. 197). The poly(A)-containing RNA is prepared as described in the published procedure with the exception that the RNA is eluted from the oligo(dT)-cellulose with water rather than SDS-containing buffer. The poly(A)-containing RNA is preciptated with ethanol and collected by centrifugation. The yield of poly(A)-containing RNA is approximately 30 µg from 300 µg of total cellular RNA. The final purified material is stored frozen at -20°C.

Example 4. Cloning of functional heavy and light chain rearrangements from the FRP5 hybridoma cell line

Poly(A)-containing RNA isolated from FRP5 hybridoma cells as described in Example 3.3 provides the source for cDNA synthesis and subsequent amplification of V-region minigenes. Amplification products of the expected size are purified from agarose gels and cloned into appropriate vectors. Functional rearrangements are identified by sequencing.

4.1 Oligonucleotides:

MCK2 is designed to be complementary to a region in the murine immunoglobulin κ (kappa) constant minigene.

5'- TCACTGGATGGTGGGAAGATGGA - 3'

MCHC2 is designed to be complementary to a region in the murine immunoglobulin γ1 constant minigene.

5'-AGATCCAGGGGCCAGTGGATAGA-3'

The oligonucleotides VH1FOR, VH1BACK, VK1FOR, and VK1BACK are designed by Orlandi et al. (Proc. Natl. Acad. Sci. USA 86: 3833, 1989) to match consensus sequences.

VH1FOR : 5' - TGAGGAGACGGTGACCGTGGTGGTCCCTTGGCCCCAG - 3'
VH1BACK: 5' - AGGT(C/G)(C/A)A(G/A)CTGCAG(G/C)AGTC(T/A)GG - 3'
VK1FOR: 5' - GTTAGATCTCCAGCTTGGT(c/G)C(C/G) - 3'
VK1BACK: 5' - GACATTCAGCTGACCCAGTCTCCA - 3'

4.2 cDNA synthesis: 55 ng of poly(A)-containing RNA is dissolved in a buffer containing 50 mM Tris-HCl, pH 8.3, 3 mM magnesium chloride, 10 mM DTT, 75 mM KCl, 400 µM dNTPs (N = G, A, T and C), 100 µg BSA (molecular biology grade, Boehringer Mannheim), 100 U RNAse inhibitor (Boehringer Mannheim), 25 pmol MCK2 and 25 pmol MCHC2. The RNA is denatured at 70°C for 5 min and then chilled on ice for 2 min. After addition of 200 U of MMLV reverse transcriptase (Gibco, BRL) cDNA synthesis is achieved by incubation for 1 h at 37°C.

4.3 Polymerase chain reaction: One tenth of the cDNA reaction is used for DNA amplification in buffer containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM β-mercaptoethanol, 200 µM dNTPs (N= G, A, T and C), 0.05 % Tween-20™ (Merck), 0.05 % NP-40™ (Merck), 10 % DMSO (Merck), 25 pmol oligonucleotide 1 (see below), 25 pmol oligonucleotide 2 (see below) and 2.5 U Amplitaq™ DNA polymerase (Perkin Elmer Cetus). Taq polymerase is added after initial denaturation at 93°C for 1 min and subsequent annealing at 37°C. In the first 4 cycles primer extension is performed at 71°C for 0.2 min, denaturation at 93°C for 0.01 min and annealing at 37°C for 0.2 min. For the last 25 cycles the annealing temperature is raised to 62°C. Finally, amplification is completed by a 3 min primer extension step at 71 °C.

PCR Product	oligonucleotide 1	oligonucleotide 2
HC	MCHC2	VH1BACK
H	VH1FOR	VH1BACK
LC	MCK2	VK1BACK
L	VK1FOR	VK1BACK

4.4 Modification and purification: Amplified material is extracted with $CHCl_3$ and precipitated with ethanol in the presence of 200 mM LiCl. To facilitate cloning, blunt ends are created by a 3 min treatment with 1 U T4

DNA polymerase (Boehringer Mannheim) in 66 mM Tris-acetate, pH 7.9, 132 mM potassium acetate, 20 mM magnesium acetate, 1 mM DTT, 200 µg/ml BSA (molecular biology grade, Boehringer Mannheim), and 400 µM dNTPs (N = G, A, T and C). The polymerase is inactivated by heating for 15 min at 65°C before phosphorylation of the DNA with 10 U T4 polynucleotide kinase (Pharmacia) at 37°C for 1 h. For this purpose the buffer is adjusted to 50 mM EDTA and 1 mM ATP. The modified amplification products are separated on a 1.2 % (w/v) agarose gel (ultra pure DNA grade agarose, Biorad) and DNA of the expected size is eluted by means of DEAE NA 45 membranes (Schleicher & Schuell).

4.5 Ligation: Bluescript™ KS+ (70 ng) linearized with XbaI, treated with Klenow DNA polymerase (Boehringer Mannheim) to give blunt ends and dephosphorylated with calf intestinal phosphatase, and 30 ng of purified amplification product are ligated using 0.5 U T4 DNA ligase (New England Biolabs) in 50 mM Tris-HCl, pH 7.8, 10 mM magnesium chloride, 10 mM DTT, and 0. 8 mM ATP overnight at 16°C. One half of the ligation mixture is used to transform E. coli K803 to obtain ampicillin resistant colonies. These are screened for the desired ligation products using a NaOH based plasmid "miniprep" method (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). The following plasmids are obtained:

PCR product	Plasmid clones
HC	pMZ15/1
	pMZ15/2
H	pMZ16/1
	pMZ16/2
L	pMZ17/1
	pMZ17/2
LC	pMZ18/1
	pMZ18/2

4.6 Sequencing: Sequencing is done using Sequenase™ kits (United States Biochemicals) with T3 and T7 oligonucleotide primers according to procedures provided by the manufacturer.

Plasmid pMZ17/1 contains a non-functional rearrangement. Plasmid pMZ 17/2 contains an Ig-unrelated sequence. Plasmids pMZ18/1 (SEQ ID NO:2) and pMZ18/2 contain identical functional FRP5 kappa light chain variable domain inserts. Plasmids pMZ16/1 (SEQ ID NO: 1) and pMZ16/2 contain identical functional FRP5 heavy chain variable domain inserts. Plasmids pMZ15/1 and pMZ15/2 also contain FRP5 heavy chain variable domain inserts together with some constant region DNA. Plasmids pMZ16/1 and pMZ18/1 are used as a source for further subcloning steps.

Example 5. Construction of the MAb FRP5 single-chain Fv gene

5.1 Construction and sequence of a cloning linker for the heavy and light chain variable domain cDNAs: Using oligonucleotides, a linker sequence which allows the cloning of PCR amplified mouse heavy chain variable domain cDNA as a PstI/BstEII fragment and of PCR amplified mouse kappa light chain variable domain cDNA as a PvuII/BglII fragment is constructed. This creates an open reading frame in which heavy and light chain variable domains are connected by a sequence coding for the 15 amino acid stretch Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser. This amino acid linker has been shown to allow correct folding of an antigen binding domain present in heavy and light chain variable domains in a single-chain Fv (Huston et al., Proc. Natl. Acad. Sci. USA 85: 5879, 1988).

For the construction of the cloning linker the 6 complementary oligonucleotides 1A, 1B, 2A, 2B, 3A, 3B are used.

1A: 5'-CAAGCTTCTCAGGTACAACTGCAGGAGGTCACCGTTTCCTCTGGGG-3'
1B: 5'-GAAACGGTGACCTCCTGCAGTTGTACCTGAGAAGTCTTGCATG-3'
2A: 5'-TGGCGGTTCTGGTGGCGGTGGCTCCGGCGGTGGCGGTTCTGAC-3'
2B: 5'-GCCACCGCCGGAGCCACCGCCACCAGAACCGCCACCGCCAGAG-3'
3A: 5'-ATCCAGCTGGAGATCTAGCTGATCAAAGCT-3'
3B: 5'-CTAGAGCTTTGATCAGCTAGATCTCCAGCTGGATGGATGTCAGAACC-3'

40 pM of oligonucleotides 1B, 2A, 2B, 3A are phosphorylated at the 5' end using T4 polynucleotide kinase (Boehringer Mannheim) in four separate reactions in a total volume of 20 µl following the method described by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). Oligonucleotides 1A and 3B are not phosphorylated in order to avoid self ligation of the linker in the final ligation reaction.

After the kinase reaction, the enzyme is inactivated by incubation at 70°C for 30 min. In three separate reactions, each containing 40 pM of two oligonucleotides in a total volume of 40 µl, non-phosphorylated 1A and phosphorylated 1B, phosphorylated 2A and phosphorylated 2B, and phosphorylated 3A and non-phosphorylated 3B are mixed. Hybridization of the oligonucleotides in the three reactions is carried out by heating to 95°C for 5 min, incubation at 65°C for 5 min and slowly cooling to room temperature. 10 µl from each of the three reactions are mixed, 4 µl of 10 x ligation buffer (Boehringer) and 4 units of T4 DNA ligase (Boehringer) are added and the total volume is adjusted to 40 µl with sterile water. The annealed pairs of oligonucleotides are ligated into one linker sequence for 16 h at 14°C. The reaction mixture is extracted with an equal volume of phenol/chloroform (1:1) followed by re-extraction of the aqueous phase with an equal volume of chloroform/isoamylalcohol (24:1). The aqueous phase is collected, 0.1 volumes of 3 M sodium acetate pH 4.8 and 2 volumes of ethanol are added, and the DNA is precipitated at -70°C for 4 h and collected by centrifugation. The resulting linker sequence has a SphI and a XbaI adaptor end. It is ligated to SphI and XbaI digested pUC19 in a reaction containing 100 ng of ligated linker and 200 ng of SphI/XbaI digested pUC19. After transformation into E. coli XL1 Blue™ (Stratagene), plasmid DNA from 4 independent colonies is isolated by the alkaline lysis mini-preparations method (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). The DNA sequence of the linker cloned in pUC19 is determined by sequencing double stranded DNA in both directions with Sequenase II (United States Biochemicals) and pUC universal and reverse primers (Boehringer) following the manufacturer's protocol. Three out of the four recombinant pUC 19 isolates sequenced contain the correct linker sequence. One of them is designated pWW 19 and used in the further experiments. The sequence is shown in SEQ ID NO:3.

5.2 Preparation of a plasmid for the subcloning of variable domains: The Fv cloning linker sequence is derived as a 144 bp HindIII/SacI fragment from pWW19 and inserted into HindIII/SacI digested Bluescript™ KS+ (ex PvuII) (Stratagene) which contains no PvuII restriction sites. The resulting plasmid, pWW15, allows cloning of heavy and light chain variable domains as PstI/BstEII and PvuII/BglII fragments, respectively.

5.2.1 Subcloning of the FRP5 heavy chain variable domain: Plasmid pMZ16/1 is digested with PstI and BstEII and the 338 bp heavy chain variable domain fragment of FRP5 is isolated. It is cloned into PstI/BstEII digested pWW19 yielding the plasmid pWW31.

5.2.2 Mutation of the FRP5 light chain variable domain and assembly of the Fv fusion gene: To facilitate subcloning of the FRP5 light chain variable domain into the Fv cloning linker, a PvuII restriction site and a BglII restriction site are introduced at the 5' and 3' ends, respectively, of the coding region. The FRP5 light chain variable domain coding region is isolated as a SacI/BamHI fragment from pMZ18/1. SacI and BamHI are restriction sites of the Bluescript™ polylinker present in pMZ18/1. The fragment contains the complete light chain variable domain fragment of 392 bp amplified by PCR using the oligonucleotide MCK2 (see above). This fragment is mutated and amplified by PCR using the oligonucleotides

V_L5':5'-GACATTCAGCTGACCAG-3' and

V_L3':5'-GCCCGTTAGATCTCCAATTTTGTCCCCGAG-3'

for the introduction of a PvuII restriction site at the 5' end (VL5') and a BglII restriction site at the 3' end (V_L3') of the kappa light chain variable domain DNA. 20 ng of the FRP5 variable light chain SacI/BamHI fragment are used as a template in a 100 µl reaction following the PCR conditions described in Example 4.3. The amplified and mutated fragment is isolated after PvuII/BglII digestion as a 309 bp fragment from a 1.5 % agarose gel and cloned into PvuII/BglII digested pWW15 generating plasmid pWW41. The FRP5 kappa light chain variable domain is isolated as a BstEII/XbaI fragment from pWW41 and inserted into BstEII/XbaI digested pWW31. Thus the FRP5 heavy chain variable domain in pWW31 and the FRP5 kappa light chain variable domain are fused to one open reading frame. Double stranded DNA of three independent clones is sequenced with Sequenase II™ kit (United Biochemicals) in both orientations using pUC universal and reverse primers (Boehringer) following the manufacturer's protocol. One of the plasmids carrying the FRP5 heavy chain variable domain fused to the mutated FRP5 light chain variable domain is selected and designated pWW52. The sequence of the HindIII/XbaI insert in plasmid pWW52 is shown in SEQ ID NO:4.

Example 6. Construction of a single-chain Fv-phosphatase fusion gene expression plasmid

The MAb FRP5 single-chain Fv gene is fused to the bacterial alkaline phosphatase. This chimeric gene encodes a bifunctional molecule which retains binding activity to the c-erbB-2 protein and has enzymatic activity.

6.1 Mutation of the single-chain Fv(FRP5) gene: To allow gene fusion between the single-chain Fv(FRP5) encoding gene from pWW52 and the alkaline phosphatase gene phoA the stop codon at sequence position 729 to 731 in pWW52 (see Example 5.2.3) is deleted as follows: Plasmid DNA of pWW52 is digested with BstEII and BglII and the linker sequence and FRP5 light chain variable domain encoding fragment is isolated. In

another digestion, pWW52 is cleaved with BstEII and BclI. Thus, the large fragment containing vector sequences and the FRP5 heavy chain variable domain encoding sequence is isolated. The BstEII/BglII V_L fragment is now inserted into BstEII/BclI cleaved pWW52 containing V_H. In the resulting plasmid, pWW53, the BglII/BclI junction is determined by sequencing double stranded DNA as described above.

Sequence of the BglII/BclI junction in pWW53 (position numbers correspond to position numbers of the HindIII/XbaI insert in plasmid pWW52, SEQ ID NO:4):

BglII/BclI

ACA AAA TTG GAG ATC AAA GCT CTA GA

714 -728 I 738 - 748

6.2 Mutation of the E.coli alkaline phosphatase gene phoA: For the construction of the Fv(FRP5)-phoA fusion gene the E. coli alkaline phosphatase gene phoA is mutated to generate a XbaI cleavage site in the coding region of phoA near the N terminus of the mature protein and a SacI cleavage site in the 3' untranslated region of phoA. This step facilitates the cloning of the mutated fragment. A pBR322 derivative carrying the recombinant transposon TnPhoA (Manoil & Beckwith, Proc. Natl. Acad. Sci. USA 82: 8129, 1985) is linearized by BglII cleavage. 20 ng of the linearized template DNA is used for a 100 µl PCR reaction carried out as described previously using oligonucleotides PhoA5' and PhoA3' as primers 1 and 2.

PhoA5':5'-CCCTCTAGAGCCTGTTCTGGAAAAC-3'

PhoA3':5'-CCCGAGCTCTGCCATTAAG-3'

Following XbaI/SacI digestion of the PCR products, a 1419 bp fragment is isolated from a 1.5 % agarose gel and inserted into XbaI/SacI digested plasmid pUC19. Ligation is carried out as described above. Ligated DNA is transformed into E. coli XL1 Blue™ (Stratagene). Thus, the open reading frame of the mutated phoA gene is fused in frame to the lacZ open reading frame of pUC19. To show that the mutated phoA gene expresses functional alkaline phosphatase, recombinant clones are plated onto LB agar plates containing 100 µg/ml ampicillin, 0.5 mM IPTG (Sigma), and 40 µg/ml XP (Boehringer). Following induction of the lac promoter of pUC 19, a lacZ-phoA fusion protein is expressed. The phosphatase activity of this fusion protein converts the indicator XP to a blue dye. One of the blue colonies is isolated and the presence of the introduced restriction sites is confirmed by digestion of miniprep DNA with XbaI and SacI. Partial 5' and 3' DNA sequences of the mutated phoA gene are obtained by sequencing double stranded DNA as described above. The DNA sequences are included in the assembly of the final Fv(FRP5)-phoA fusion gene sequence shown in SEQ ID NO:5. The isolated plasmid is designated pWW61 and used for further subcloning steps.

6.3 Construction of a FRP5 Fv-phoA expression plasmid: From plasmid pWW19 (see Example 5.1.2) the cloning linker sequence is isolated as a HindIII/EcoRI fragment and inserted into HindIII/EcoRI digested plasmid pIN III-ompA-Hind (Rentier-Delrue et al., Nucl. Acids Res. 16: 8726, 1988) leading to plasmid pWW16.

From pWW61 (see Example 6.2) the mutated phoA gene is isolated as a XbaI/SacI fragment and inserted into XbaI/SacI digested pWW53. The resulting plasmid, pWW615, carries the Fv(FRP5) gene fused in frame to the mutated alkaline phosphatase gene. The Fv(FRP5)-phoA gene is isolated as a HindIII/SacI fragment from pWW615 and inserted into HindIII/SacI digested plasmid pWW16. This leads to the production of the Fv(FRP5)-phoA expression plasmid pWW616 (see below). All ligations are carried out as described above. Recombinant plasmids are transformed into E. coli XL1 Blue™ (Stratagene). The constructs are confirmed by restriction enzyme analysis of plasmid DNA isolated by an alkaline mini preparation method (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982).

In this construct the Fv single-chain antibody of FRP5, genetically fused to the alkaline phosphatase phoA, can be expressed in E. coli following induction with IPTG. The recombinant protein carries the E. coli outer membrane protein A (ompA) signal sequence at the N terminus (encoded by the pIN III-ompA-Hind vector) to facilitate secretion of the protein into the periplasmic space of E. coli expressor cells.

The sequence of the Fv(FRP5)-phoA fusion gene in expression plasmid pWW616 is shown in SEQ ID NO:5. Part of the phoA sequence is assembled from Chang et al., Gene 44: 121, 1986.

Example 7. Expression of Fv(FRP5)-phoA in E. coli

Plasmid pWW616 is transformed into the phoA negative E. coli strain CC118 (Manoil & Beckwith, Proc. Natl. Acad. Sci. USA 82: 8129, 1985). A recombinant single colony is grown overnight in 50 ml LB medium containing 70 µg/ml ampicillin. The overnight culture is diluted 1:10 in 500 ml fresh LB medium containing 70 µg/ml ampicillin and grown at 37°C to an OD_{550} of 0.1. IPTG is added to a final concentration of 2 mM and exp-

ression is induced for 1.5 h at 37°C. The cells are harvested at 4°C by centrifugation at 4000 rpm for 25 min in a Beckman GPKR centrifuge. The supernantant of CC118/pWW616 is set aside on ice for preparation of Fv(FRP5)-phoA, see Example 7.2.

7.1 Isolation of Fv(FRP5)-phoA from the periplasmic proteins of CC118/pWW616: The bacterial pellet is suspended in 10 ml TES buffer (0.2 M Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 M sucrose) and kept on ice for 10 min. After centrifugation at 4°C for 10 min at 5000 rpm in a Heraeus minifuge, the supernatant is discarded and the washed pellet is suspended in 15 ml ice-cold TES, diluted (1:4) with water. The cells are kept on ice for 30 min and recentrifuged as above. The supernatant containing periplasmic proteins is recentrifuged at 45,000 x g for 15 min in a Beckman TL100 ultracentrifuge. The periplasmic extract is concentrated in an Amersham ultrafiltration unit through a YM10 membrane to a final volume of 2 ml. Following fivefold dilutions with PBS and reconcentration through the YM10 membrane five times, the 1:4 diluted TES buffer of the periplasmic extract is exchanged with PBS. NaN_3 and protease inhibitors are added to the periplasmic proteins (2 ml in PBS) to the final concentration of 0.02 % NaN_3, 0.1 mM PMSF, 2 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin. The periplasmic extract is stored at 4°C.

7.2 Isolation of Fv(FRP5)-phoA from the concentrated supernatant of E. coli CC118/pWW616 cultures: The supernatant (500ml) of the induced E. coli culture CC118/pWW616 is filtered through a 0.45 µm membrane. The filtrate is concentrated in an Amicon ultrafiltration unit through a YM10 membrane to a final volume of 10 ml in PBS as described above. NaN_3 and protease inhibitors are added to the concentrated supernatant to the final concentrations indicated above. The concentration of Fv(FRP5)-phoA in the extracts is determined by densitometry in comparison to BSA standards of coomassie stained 9 % SDS-PAGE gels.

Example 8. Activity of Fv(FRP5)-phoA

8.1 Detection of c-erbB-2 in SKBR3 breast tumor cells by immunostaining using Fv(FRP5)-phoA: The Fv domain of Fv(FRP5)-phoA enables the molecule to bind to the extracellular domain of the c-erbB-2 protein. Bound Fv(FRP5)-phoA can be visualized by staining procedures using color substrates for the detection of alkaline phosphatase activity.

8.1.1 Fixation of cells: SKBR3 human breast tumor cells carrying about 1 x 10^6 c-erbB-2 receptors per cell are grown on fibronectin coated glass cover slips. The cells are washed twice with PBS and then fixed with PBS / 3.7 % formaldehyde at room temperature for 30 min. The fixed cells are washed three times with PBS at room temperature. Unspecific binding sites are blocked by incubating the cells for 1 h with PBS / 3 % BSA at 37°C in a humid incubator. The cells are then washed twice with PBS.

8.1.2 Pretreatment of Fv(FRP5)-phoA: Alkaline phosphatase phoA from E. coli must be dimerized to be enzymatically active. In the periplasm of E. coli natural phoA is dimerized, i.e. two molecules of phoA are held together by two Zn^{2+} ions. The Fv(FRP5)-phoA is also produced as a dimer in E. coli. To increase binding of Fv(FRP5)-phoA to the antigen, the dimers are monomerized by adding EGTA to the solution. This step removes Zn^{2+} from the solution. Monomerized phosphatase can be re-dimerized by the addition of Zn^{2+}. EGTA is added to a final concentration of 5 mM to 200 µl of 40 x concentrated supernatant or periplasmic proteins from CC118/pWW616 (see above). The solution is incubated at 37°C for 1 h just before use in the immunoassay.

8.1.3 Staining of cells: After blocking with PBS / 3 % BSA (see above) fixed cells are incubated for 1 h with pretreated Fv(FRP5)-phoA at a concentration of 1 µg/ml at 37°C in a humidified incubator. The cells are washed three times with PBS at room temperature. The staining solution consists of 300 µl naphtol AS-MX™ phosphate (Sigma, 13 mg/ml in dimethyl formamide), 8 mg of levamisole (Sigma), and 10 mg of Fast Red TR™ salt (Sigma) added to 9.7 ml of 100 mM Tris-HCl, pH 8.2, 1 mM $ZnCl_2$. This mixture is prepared and filtered through a 0.45 µm filter immediately before use. $ZnCl_2$ is added to the staining solution to allow re-dimerization of bound Fv(FRP5)-phoA and thereby activating the alkaline phosphatase. Cells are incubated in the Fast Red™ staining solution for 15 min at room temperature. The phosphatase activity is blocked after staining by washing the cells twice with PBS and once with 1 M KH_2PO_4. Glass cover slips are mounted with gel mount (Biomeda). The cells are examined under a fluorescence microscope using green light for excitation. Stained SKBR3 cells show intense red cell surface fluorescence.

8.2 Detection of c-erbB-2 protein over-expression in immunoblots using Fv(FRP5)-phoA: Proteins from total cell lysates of SKBR3 cells over-expressing c-erbB-2 protein are separated by SDS-PAGE and blotted onto PVDF membrane (Millipore). For preparation of extracts and immunoblotting technique see Example 1.3.2. Free binding sites of the membrane are blocked by incubation for 1 h at room temperature in a solution containing 10 mM Tris-HCl, pH 7.5, 0.9 % NaCl, 0.05 % Tween 20™ (BioRad), and 3 % BSA. Pretreated Fv(FRP5)-phoA (see Example 7.2.) is diluted in blocking solution to a final concentration of 0.1 µg/ml. The membrane is incubated in the Fv(FRP5)-phoA solution for 1 h at room temperature and then washed three times for 5 min at room temperature in 10 mM Tris-HCl, pH 7.5, 0.9 % NaCl, 0.05 % Tween 20™ and once in 10 mM Tris-HCl,

pH 7.5, 0.9 % NaCl. For detection of bound Fv(FRP5)-phoA the membrane is incubated for 20 min at 37°C in the Fast Red™ substrate solution described in Example 7.3 without levamisole. The reaction is stopped by washing the membrane twice in water. Fv(FRP5)-phoA specifically detects the 185 kD c-erbB-2 protein.

Example 9. Expression and isolation of Fv(FRP5)-phoA from E. coli

9.1 Preparation of periplasmic extract: Plasmid pWW616 is transformed into the phoA negative E. coli strain CC118 according to standard procedures (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). A single colony is picked and grown overnight in LB medium containing 70 µg/ml ampicillin. The overnight culture is diluted 1:10 in fresh LB medium contalning ampicillin and grown at 37°C to an OD_{550} of 0.1. At this point expression of the Fv(FRP5)-phoA gene is induced by the addition of IPTG to a final concentration of 2 mM, and the cells are grown for an additional 1.5 to 2 h. The cells are harvested by centrifugation and treated with a mild osmotic shock which releases the periplasmatic proteins into the supernatant. The proteins are concentrated in an Amersham ultrafiltration unit through a YM10 membrane.

9.2 Preparation of an antigen affinity column: The c-erbB-2 protein is isolated from insect cells infected with a baculovirus vector expressing the c-erbB-2 extracellular domain by standard methods (V.A. Luckow & M.D. Summers, Biotechnology 6: 47-55, 1988). MAb FSP77 is coupled to CNBR-activated Sepharose 4B™ (Pharmacia) following the instructions of the manufacturer. The insect cell lysates are incubated with the coupled MAb FSP77 in a buffer containing 50 mM Tris-HCl, pH 7.5,5 mM EGTA, 0.5 % Triton X-100™, 150 mM NaCl for 2 h at 4°C on a shaking platform. The beads are packed into a column and washed with pre-elution bufter consisting of 10 mM phosphate, pH 6.8, and 100 mM NaCl to remove non-specifically bound proteins. The c-erbB-2 protein is recovered from the column by treatment with a low pH elution buffer containing 100 mM glycine, pH 3.0, and 100 mM NaCl. The fractions from the column are collected into phosphate buffer, pH 8.0, in order to raise the pH. The c-erbB-2 extracellular domain is detected by running a part of each fraction on 8 % SDS-PAGE gel, blotting onto PVDF membrane (Millipore) and treating the filter with MAb FSP77 followed by sheep anti-mouse IgG. Bound IgG is detected by ^{125}I-Protein-A treatment. The fractions containing the extracellular domain are pooled and the protein is coupled to CNBR-activated Sepharose 4BTM (Pharmacia) following the instructions of the manufacturer.

9.3 Isolation of Fv(FRP5)-phoA by affinity chromatography: The sepharose coupled to c-erbB-2 protein (Examle 9.2) is incubated for 2-4 h at 4°C on a rocking platform with the periplasmic extract isolated as described in Example 9.1. The beads are packed into a column and washed with pre-elution buffer as in Example 9.2. The Fv(FRP5)-phoA protein is recovered by elution with the low pH elution buffer of Example 9.2. The fractions are monitored for the presence of the Fv(FRP5)-phoA by testing for phoA enzymatic activity using a standard protocol.

Example 10. Immunoassay for c-erbB-2 protein in tumors

10.1 Preparation of tumor sections: To determine the level of c-erbB-2 protein in tumors, tumor tissue is pretreated to give either frozen tumor sections or paraffin-embedded tumor sections. Tumor pieces are quick frozen, then cut with a cryostat, collected onto 1 % gelatin-coated glass slides, and fixed with 4 % paraformaldehyde. Following several washes with PBS, the tumor tissue sections are ready for staining. Alternatively, tumor pieces are placed in 4 % paraformaldehyde for fixation, embedded in paraffin, then sections cut and collected onto polylysine-coated glass cover slips. To prepare the sections for staining, they are heated overnight at 56°C, dewaxed in xylene, stepwise rehydrated by washing in 95 %, 70 % and 35 % ethanol and water, and washed in PBS.

10.2 Pretreatment of Fv(FRP5)-phoA: Since the dimer of the Fv(FRP5)-phoA as obtained from the E. coli periplasm does not bind optimally to the c-erbB-2 antigen, it is first monomerized. This is accomplished by treating the solution of Fv(FRP5)-phoA for 1 h at 37°C with EGTA at a final concentration of 5 mM. This treatment chelates the Zn^{2+} ions which are important for maintaining the dimeric structure of Fv(FRP5)-phoA.

10.3 Staining of the tumor sections: Non-specific staining of the tumor sections prepared according to Example 10.1 is blocked by incubating the sections in PBS containing 3 % BSA. The blocked sections are incubated for 1 - 2 h with pretreated Fv(FRP5)-phoA (Example 10.2) at a concentration of 1 µg/ml in a humidified chamber at room temperature. The sections are washed three times with PBS at room temperature. The bound Fv(FRP5)-phoA protein is detected using Fast Red™ as a substrate for the alkaline phosphatase. The staining solution consists of 300 µl naphthol AS-MX phosphate (Sigma, 13 mg/ml in dimethylformamide), 8 mg of levamisole (an inhibitor of endogenous alkaline phosphatase, Sigma), and 10 mg of Fast Red TR™ salt (Sigma) added to 9.7 ml of 100 mM Tris-HCl, pH 8.2, and 1 mM $ZnCl_2$. This mixture is prepared and filtered through a 0.45 µm filter immediately before use. $ZnCl_2$ is added to the staining solution to allow re-dimerization of the bound

Fv(FRP5)-phoA protein and activation of the alkaline phosphatase. The tumor sections treated with Fv(FRP5)-phoA are incubated in the Fast Red™ staining solution for 15 min at room temperature. After staining the phosphatase activity is blocked by washing the cells twice with PBS and once with 1 M KH_2PO_4. The glass cover slips are mounted with gel mount. The cells are examined under a fluorescence microscope using green light for excitation. Positively stained cells show an intense red cell surface fluorescence.

Alternatively, the tumor sections treated with the Fv(FRP5)-phoA protein may be stained with naphthol AS-BI phosphate (Sigma) and New Fuchsin™ (Sigma), or with 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) and Nitro Blue Tetrazolium™ (Sigma). The stained sections can then be viewd with a regular light microscope.

Example 11. Cloning of functional heavy and light chain rearrangements from the FWP51 hybridoma cell line

Poly(A)-containing RNA isolated from FWP51 hybridoma cells as described in Example 3.3 provides the source for cDNA synthesis and subsequent amplification of V-region minigenes. cDNA synthesis and amplification of FWP51 heavy and light chain variable domain cDNA by polymerase chain reaction is carried out as described in Example 4. Amplification products of the expected size are purified from agarose gels and cloned into appropriate vectors. Functional rearrangements are identified by sequencing.

11.1 Subcloning of FWP51 heavy and light chain variable domain cDNA: Material amplified according to Example 4.3 is extracted with $CHCl_3$ and precipitated in the presence of 200 mM LiCl. To facilitate cloning, the FWP51 heavy chain variable domain cDNA is cleaved with restriction enzymes PstI and BstEII, the fragment purified by agarose gel electrophoresis, and ligated to PstI and BstEII digested pWW15 DNA. The FWP51 light chain variable domain cDNA is cleaved with restriction enzymes PvuII and BglII, the fragment is purified by agarose gel electrophoresis, and ligated to PvuII and BglII digested pWW15 DNA (cf. Example 5). Ligation, transformation, and screening for the desired ligation products are carried out as described in Example 4.5. The following plasmids are obtained:

PCR product	Plasmid clones
H	pWW15-VH51-1
	pWW15-VH51-2
	pWW15-VH51-3
L	pWW15-VL51-1
	pWW15-VL51-2
	pWW15-VL51-3

11.2 Sequencing: Sequencing is done as described in Example 4.6.

Plasmids pWW15-VH51-1 (SEQ ID NO:6), pWW15-VH51-2, pWW15-VH51-3 contain identical functional FWP51 heavy chain variable domain inserts. Plasmids pWW15-VL51-1 (SEQ ID NO:7), pWW15-VL51-2, pWW15-VL51-3 contain identical functional FWP51 kappa light chain variable domain inserts. Plasmids pWW15-VH51-1 and pWW15-VL51-1 are used as a source for further subcloning steps.

Example 12. Construction of the MAb FWP51 single chain gene

12.1 Assembly of the Fv fusion gene: Plasmid pWW15-VH51-1 is digested with PstI and BstEII and the 342 bp heavy chain variable domain fragment of FWP51 is isolated. It is cloned into PstI/BstEII digested pWW15-VL-51-1 yielding the plasmid pWW15-Fv51 (SEQ ID NO: 8).

12.2 Mutation of the single-chain Fv(FWP51) gene: To allow gene fusion between the single-chain Fv(FWP51) encoding gene from pWW15-Fv51 and effector genes the stop codon at sequence position 729 to 731 in pWWFv15-51 (SEQ ID NO:8) is deleted as follows (see also Example 6.1): plasmid DNA of pWW15-Fv51 is digested with BstEII and BglII and the linker sequence and FWP51 light chain variable domain encoding fragment is isolated. In another digestion, pWW15-Fv51 is cleaved with BstEII and BclI. Thus, the large fragment containing vector sequences and the FWP51 heavy chain variable domain encoding sequence is isolated. The

BstEII/BglII VL fragment is now inserted into BstEII/BclI cleaved pWW15-Fv51 containing V_H. The resulting plasmid pWW15-Fv51-ORF is used as a source for the construction of Fv(FWP51)-effector fusion genes.

Example 13. Construction of single-chain Fv-exotoxin A fusion gene expression plasmids

The MAb FRP5 and MAb FWP51 single-chain Fv genes are fused to a truncated bacterial toxin, exotoxin A (ETA) from Pseudomonas aeruginosa. These chimeric genes encode recombinant immunotoxins which selectively inhibit protein synthesis in c-erbB-2 expressing cells.

13.1 Mutation of the Exotoxin A gene of Pseudomonas aeruginosa PAK: For the construction of Fv-exotoxin A (Fv-ETA) fusion genes the ETA gene from Pseudomonas aeruginosa PAK is mutated to delete the original cell binding domain I at the N-terminus of the toxin and to generate a XbaI cleavage site at the former domain I/domain II boundary of the ETA coding region. Plasmid pMS150A (Lory et al., J. Bacteriol. 170: 714, 1988) is linearized by EcoRI cleavage. 20 ng of the linearized template DNA is used for a 100 µl PCR reaction carried out as described previously using the following oligonucleotides as primers 1 and 2.

1: 5'-CACGGAAGCTTAAGGAGATCTGCATGCTTCTAGAGGGCGGCA-
GCCTGGCCGCGCTG-3'

2: 5'-GCGGATCGCTTCGCCCAGGT-3'

Following HindIII/SalI digestion of the PCR products, a 201 bp fragment is isolated from a 1.5% agarose gel and inserted into HindIII/SalI digested plasmid pUC18. Ligation is carried out as described above. Ligated DNA is transformed into E.coli XL1 Blue™ (Stratagene). Two recombinant plasmids are isolated and the insert DNA is sequenced as described above using pUC universal and reverse primers (Boehringer). One plasmid containing the expected product is designated pWW22 (SEQ ID NO:9) and used as a source for further subcloning steps. Plasmid pWW22 is cleaved with HindIII and SalI, the mutated ETA gene fragment is isolated, and inserted into the large fragment of HindIII/SalI digested plasmid pMS150A containing pUC9 vector sequences and part of the ETA gene coding for the C-terminal half of the toxin. Thereby in the resulting plasmid pWW20 a truncated ETA gene coding for domains II and III of the toxin is created.

13.2 Assembly of single-chain Fv-ETA fusion genes: HindIII/XbaI single-chain Fv gene fragments suitable for the construction of Fv-ETA fusion genes are isolated from plasmid pWW53 (single-chain Fv FRP5), and plasmid pWW15-Fv51-ORF (single-chain Fv FWP51) and inserted into HindIII/XbaI digested pWW20. Ligation and transformation into E.coli XL1 Blue™ (Stratagene) are carried out as described above. The resulting plasmids pWW20-Fv5 (Fv(FRP5)-ETA) and pWW20-Fv51 (Fv(FWP51)-ETA) are used as a source for further subcloning steps.

13.3 Construction of single-chain Fv-exotoxin A fusion gene expression plasmids: For the expression of single-chain Fv-exotoxin A fusion genes in E.coli the expression plasmid pFLAG-1 (IBI Biochemicals) is used. The fusion-genes are fused in frame to the outer membrane protein A (ompA) signal sequence encoded by pFLAG-1. Plasmid DNA from pWW20-Fv5 and pWW20-Fv51 is digested with HindIII and blunt ends are created by Klenow fill-in as described in Example 4.5. Blunt ended DNA is digested with EcoRI and single-chain Fv-ETA gene fragments are isolated (Fv(FRP5)-ETA: 1916 bp, Fv(FWP51)-ETA: 1916 bp). pFLAG-1 plasmid DNA is digested with HindIII, blunt ends are created as described above, the resulting DNA fragment is isolated, and digested with EcoRI. Blunt-end/EcoR1 Fv-ETA fusion gene fragments are inserted into the modified pFLAG-1 plasmid DNA. Thereby Fv-ETA fragments are fused in frame to the ompA signal sequence of pFLAG-1 creating plasmids pWW215-5 for the expression of Fv(FRP5)-ETA (SEQ ID NO: 10) and pWW215-51 for the expression of Fv(FWP51)-ETA (SEQ ID NO: 11).

Example 14. Expression and Isolation of Fv(FRP5)-ETA and Fv(FWP51)-ETA from E. coli

14.1 Preparation of total lysates: Plasmids pWW215-5 and pWW215-51 are transformed into the E.coli strain CC118 according to standard procedures (see Example 9.1). Single colonies are picked and grown overnight in LB medium containing 100 µg/ml ampicillin and 0.4% glucose. The overnight cultures are diluted 1:30 in fresh LB medium containing ampicillin and glucose and grown at 37°C to an OD_{550} of 0.5. At this point expression of the Fv(FRP5)-ETA and Fv(FWP51)-ETA genes is induced by the addition of IPTG to a final concentration of 0.5 mM, and the cells are grown for an additional 30 min. The cells are harvested by centrifugation and lysed by sonication in PBS/ 1 mM $CaCl_2$. The lysates are cleared by ultracentrifugation at 25 000 g for 45 min at 4°C. The supernatants are collected.

14.2 Isolation of Fv(FRP5)-ETA and Fv(FWP51)-ETA by affinity chrometography: Cleared E.coli lysates conteining the 66.4 kDa Fv(FRP5)-ETA or the 66.3 kDa Fv(FWP51)-ETA protein are passed through a M1 monoclonal antibody affinity column (IBI Biochemicals). The column is washed three times with PBS/1 mM $CaCl_2$. Bound Fv(FRP5)-ETA or Fv(FWP51)-ETA proteins are eluted with PBS/2 mM EDTA. The fractions ere monitored for the presence of Fv-ETA proteins by SDS-PAGE and immunoblotting (see Example 1.3.2) using an anti-exotoxin A antiserum developed in rabbit.

Example 15. Selective inhibition of protein synthesis in c-erbB-2 expressing cells with Fv(FRP5)-ETA and Fv(FWP51)-ETA

In vitro the recombinant immunotoxins Fv(FRP5)-ETA and Fv(FWP51)-ETA selectively inhibit protein synthesis and growth of cells expressing high levels of the human c-erbB-2 protein. The immunotoxins do not affect cells expressing no, or low levels of human c-erbB-2 protein.

15.1 Immunotoxin treetment of cell lines: Human breast end overian tumor cell lines SK-BR3, MDAMB-231, MDA-MB-453, HTB77, the mouse mammary epithelial cell line HC11, and HC11 cells transfected with the human c-erbB-2 cDNA ere plated on 48 well tissue culture pletes (Costar) at e density of 10^5 cells/well. After 4 h the medium is removed and replaced by normal growth medium contalning Fv(FRP5)-ETA or Fv(FWP51)-ETA at various concentrations ranging from 1 to 1000 ng/ ml. The cells are incubated with toxin fusion proteins for 16 h.

15.2 ^{3}H-leucine labeling of cells: The immunotoxin-treated cells are washed twice end incubated In normal growth medium containing 4 µCi ^{3}H-leucin/ ml for 4 h. The lebeled cells are washed twice end ^{3}H-leucine lebeled total proteins are harvested by TCA precipitation onto Whatman GFC filters. The rate of protein synthesis in immunotoxin-treated cells is determined in comparison to untreated control cells.

Example 16: Fv(FRP5)-ETA and MAbs FWP51 and FSP77 Inhibit the growth of c-erbB-2 expressing cells in nude mice.

The administration of Fv(FRP5)-ETA and the MAbs FWP51 and FSP77 to animals injected with c-erbB-2 expressing cells inhibits the tumor growth of these cells.

16.1 Nude mouse tumor model.: The NIH/3T3 mouse fibroblast cell line is transfected according to conventional, previously described methods (Graham & van der Eb, Virology 52: 456, 1973) with a plasmid expressing the point mutated, activated human c-erbB-2 protein (Masuko et al., Jpn. Cancer Res. 80: 10, 1989) and with the plasmid pSV2neo (Southern & Berg, J. Mol. Appl. Genet. 1:327, 1982) which encodes the gene for resistance to the drug G418. Transfected cells are selected 2 weeks in medium conteining 500 ug/ml G418 (Geneticin, Gibco-BRL). Individual clones are selected and enelyzed for the expression of the human c-erbB-2 protein using conventional protein blotting techniques (Towbin et el., Proc. Natl. Acad. Scl. USA 76: 4350, 1979). A clone expressing moderate levels of the point mutated, ectivated human c-erbB-2 protein (clone 3.7) is selected, and tested for growth in nude mice. 2-5 x 106 clone 3.7 cells (per animal) suspended in 0.2 ml PBS ere subcutaneously injected into the flank of female Belb/c nude mice. The 3.7 cells injected at e dose of 2 x 106 cells rapidly form tumors in nude mice (control animals, cf. Example 16.2)

16.2 Immunotoxin treatment of animals: 2 x 106 clone 3.7 cells are injected subcutaneously into nude mice. The animals are treated continuously for a total of 7 deys with the Fv(FRP5)-ETA. 200 µl of Fv(FRP5)-ETA (concentration 35 µg/ml in PBS) is placed in an osmotic pump (Alzet mini osmotic pump, Model 2001, Alze, Pelo Alto, CA, #94303-0802) which is implented subcutaneously into the animals at the seme time as the clone 3.7 cells are injected. The pump continuously releases Fv(FRP5)-ETA and delivers 1 µg/day for 7 days to each animal. In comparison with the control enimels (cf. Example 16.1), the edministration of Fv(FRP5)-ETA delays the onset of tumor formation.

16.3 MAb treetment of animals: 5 x 106 clone 3.7 cells are injected subcutaneously into nude mice. Starting on the same day as injection of clone 3.7 cells, the animals are treeted daily, for a total of 10 days, with either MAb FWP51 or MAb FSP77 (MAb dose Is 50 ug/200 ul BSS/day). The MAb is injected intraveneously in the tail vein of the mouse. Both antibodies delay the onset of tumor growth. Compared therewith, a synergistic effect in inhibiting tumor growth is observed on simultaneous administration of both antibodies MAb FWP51 end MAb FSP77.

Sequence listing

SEQ ID NO:1

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 361 bp
MOLECULE TYPE: plasmid DNA
ORIGINAL SOURCE ORGANISM: mouse
IMMEDIATE EXPERIMENTAL SOURCE: E. coli
NAME OF CELL CLONE: pMZ16/1

FEATURES:		
	from 6 to 27 bp	VH1BACK primer region
	from 95 to 109 bp	CDR_{1H}
	from 152 to 202 bp	CDR_{2H}
	from 299 to 328 bp	CDR_{3H}
	from 329 to 361 bp	VH1FOR primer region

PROPERTIES: encodes the heavy chain variable domain of monoclonal antibody FRP5

```
TCTAGAGGTG AAACTGCAGC AGTCTGGACC TGAACTGAAG AAGCCTGGAG  50

AGACAGTCAA GATCTCCTGC AAGGCCTCTG GGTATCCTTT CACAAACTAT 100

GGAATGAACT GGGTGAAGCA GGCTCCAGGA CAGGGTTTAA AGTGGATGGG 150

CTGGATTAAC ACCTCCACTG GAGAGTCAAC ATTTGCTGAT GACTTCAAGG 200

GACGGTTTGA CTTCTCTTTG GAAACCTCTG CCAACACTGC CTATTTGCAG 250

ATCAACAACC TCAAAAGTGA AGACATGGCT ACATATTTCT GTGCAAGATG 300

GGAGGTTTAC CACGGCTACG TTCCTTACTG GGGCCAAGGG ACCACGGTCA 350

CCGTCTCCTC A                                           361
```

SEQ ID NO:2

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 407 bp
MOLECULE TYPE: plasmid DNA
ORIGINAL SOURCE ORGANISM: mouse
IMMEDIATE EXPERIMENTAL SOURCE: E. coli
NAME OF CELL CLONE: pMZ18/1

FEATURES:		
	from 6 to 28 bp	MCK2 primer region
	from 98 to 130 bp	CDR_{1L}
	from 176 to 196 bp	CDR_{2L}
	from 293 to 319 bp	CDR_{3L}
	from 374 to 404 bp	MCK2 primer region

PROPERTIES: encodes the kappa light chain variable domain of monoclonal antibody FRP5

```
TCTAGTCACT GGATGGTGGG AAGATGGAGA CATTGTGATG ACCCAGTCTC  50

ACAAATTCCT GTCCACTTCA GTAGGAGACA GGGTCAGCAT CACCTGCAAG 100

GCCAGTCAGG ATGTGTATAA TGCTGTTGCC TGGTATCAAC AGAAACCAGG 150

ACAATCTCCT AAACTTCTGA TTTACTCGGC ATCCTCCCGG TACACTGGAG 200

TCCCTTCTCG CTTCACTGGC AGTGGCTCTG GGCCGGATTT CACTTTCACC 250

ATCAGCAGTG TGCAGGCTGA AGACCTGGCA GTTTATTTCT GTCAGCAACA 300

TTTTCGTACT CCATTCACGT TCGGCTCGGG GACAAAATTG GAAATAAAAC 350

GGGCTGATGC TGCACCAACT GTATCCATCT TCCCACCATC CAGTGACTAG 400

AACTAGA                                                407
```

SEQ ID NO:3

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 175 bp
MOLECULE TYPE: plasmid DNA
ORIGINAL SOURCE ORGANISM: fully synthetic
IMMEDIATE EXPERIMENTAL SOURCE: E. coli
NAME OF CELL CLONE: pWW19

FEATURES:		
	from 30 to 35 bp	PstI site
	from 38 to 44 bp	BstEII site for subcloning of heavy chain variable domain
	from 54 to 98 bp	coding sequence of $(GlyGlyGlyGlySer)_3$ linker
	from 105 to 110 bp	PvuII site
	from 112 to 117 bp	BglII site
	from 120 to 125 bp	BclI site for subcloning of light chain variable domain

```
AAGCTTGCAT GCAAGCTTCT CAGGTACAAC TGCAGGAGGT CACCGTTTCC   50

TCTGGCGGTG GCGGTTCTGG TGGCGGTGGC TCCGGCGGTG GCGGTTCTGA  100

CATCCAGCTG GAGATCTAGC TGATCAAAGC TCTAGAGGAT CCCCGGGTAC  150

CGAGCTCGAA TTCACTGGCC GTCGT                              175
```

SEQ ID NO:4

SEQUENCE TYPE: nucleotide with corresponding protein
SEQUENCE LENGTH: 748 bp
MOLECULE TYPE: plasmid DNA
ORIGINAL SOURCE ORGANISM: mouse
IMMEDIATE EXPERIMENTAL SOURCE: E. coli
NAME OF CELL CLONE: pWW52

FEATURES:		
	from 1 to 8 bp	synthetic spacer
	from 9 to 365 bp	FRP5 heavy chain variable domain
	from 99 to 113 bp	CDR_{1H}
	from 156 to 206 bp	CDR_{2H}
	from 303 to 332 bp	CDR_{3H}
	from 366 to 410 bp	15 amino acids linker sequence
	from 411 to 728 bp	FRP5 light chain variable domain
	from 480 to 512 bp	CDR_{1L}
	from 558 to 578 bp	CDR_{2L}
	from 675 to 701 bp	CDR_{3L}

PROPERTIES: Fv heavy chain/light chain variable domain fusion protein binding to the extracellular domain of the growth factor receptor c-erbB-2

```
AAGCT TCT CAG GTA CAA CTG CAG CAG TCT GGA CCT GAA CTG       41
      Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu
                        5                  10

AAG AAG CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GCC TCT    83
Lys Lys Pro Gly Glu Thr Val Lys Ile Ser Cys Lys Ala Ser
         15                 20                 25

GGG TAT CCT TTC ACA AAC TAT GGA ATG AAC TGG GTG AAG CAG   125
Gly Tyr Pro Phe Thr Asn Tyr Gly Met Asn Trp Val Lys Gln
             30                 35                 40

GCT CCA GGA CAG GGT TTA AAG TGG ATG GGC TGG ATT AAC ACT   167
Ala Pro Gly Gln Gly Leu Lys Trp Met Gly Trp Ile Asn Thr
                   45                 50
```

```
TCC ACT GGA GAG TCA ACA TTT GCT GAT GAC TTC AAG GGA CGG   209
Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp Phe Lys Gly Arg
 55                  60                  65

TTT GAC TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT TTG   251
Phe Asp Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala Tyr Leu
     70                  75                  80

CAG ATC AAC AAC CTC AAA AGT GAA GAC ATG GCT ACA TAT TTC   293
Gln Ile Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr Phe
         85                  90                  95

TGT GCA AGA TGG GAG GTT TAC CAC GGC TAC GTT CCT TAC TGG   335
Cys Ala Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr Trp
            100                 105                 110

GGC CAA GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT GGC GGT   377
Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGC GGT GGC TCC GGC GGT GGC GGT TCT GAC ATC CAG   419
Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln
125                 130                 135

CTG ACC CAG TCT CAC AAA TTC CTG TCC ACT TCA GTA GGA GAC   461
Leu Thr Gln Ser His Lys Phe Leu Ser Thr Ser Val Gly Asp
    140                 145                 150

AGG GTC AGC ATC ACC TGC AAG GCC AGT CAG GAT GTG TAT AAT   503
Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Tyr Asn
        155                 160                 165

GCT GTT GCC TGG TAT CAA CAG AAA CCA GGA CAA TCT CCT AAA   545
Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys
            170                 175                 180
```

```
CTT CTG ATT TAC TCG GCA TCC TCC CGG TAC ACT GGA GTC CCT   587
Leu Leu Ile Tyr Ser Ala Ser Ser Arg Tyr Thr Gly Val Pro
                185                 190

TCT CGC TTC ACT GGC AGT GGC TCT GGG CCG GAT TTC ACT TTC   629
Ser Arg Phe Thr Gly Ser Gly Ser Gly Pro Asp Phe Thr Phe
195                 200                 205

ACC ATC AGC AGT GTG CAG GCT GAA GAC CTG GCA GTT TAT TTC   671
Thr Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Phe
    210                 215                 220

TGT CAG CAA CAT TTT CGT ACT CCA TTC ACG TTC GGC TCG GGG   713
Cys Gln Gln His Phe Arg Thr Pro Phe Thr Phe Gly Ser Gly
        225                 230                 235

ACA AAA TTG GAG ATC TAGCTGATCA AAGCTCTAGA                 748
Thr Lys Leu Glu Ile
            240
```

SEQ ID NO:5

SEQUENCE TYPE: nucleotide with corresponding protein
SEQUENCE LENGTH: 2233 bp
MOLECULE TYPE: plasmid DNA
ORIGINAL SOURCE ORGANISM: mouse and E. coli
IMMEDIATE EXPERIMENTAL SOURCE: E. coli
NAME OF CELL CLONE: pWW616

FEATURES:	
from 1 to 22 bp	ompA 5' non-coding region
from 23 to 85 bp	ompA signal peptide
from 89 to 445 bp	FRP5 heavy chain variable domain
from 446 to 490 bp	15 amino acids linker sequence
from 491 to 814 bp	FRP5 light chain variable domain
from 815 to 2155 bp	coding region of phoA
from 2156 to 2233 bp	3' non-coding region of phoA

PROPERTIES: Fv heavy chain/light chain variable domain and alkaline phosphatase fusion protein Fv(FRP5)-phoA binding to the growth factor receptor c-erbB-2

```
TCTAGATAAC GAGGCGCAAA AA    ATG AAA AAG ACA GCT ATC GCG     43
                            Met Lys Lys Thr Ala Ile Ala
                               -20                 -15

ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAA GCT  85
Ile Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala
            -10                 -5

TCT CAG GTA CAA CTG CAG CAG TCT GGA CCT GAA CTG AAG AAG  127
Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Lys Lys
 1              5                  10

CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GCC TCT GGG TAT  169
Pro Gly Glu Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr
 15             20                  25

CCT TTC ACA AAC TAT GGA ATG AAC TGG GTG AAG CAG GCT CCA  211
Pro Phe Thr Asn Tyr Gly Met Asn Trp Val Lys Gln Ala Pro
     30             35                  40
```

```
GGA CAG GGT TTA AAG TGG ATG GGC TGG ATT AAC ACC TCC ACT   253
Gly Gln Gly Leu Lys Trp Met Gly Trp Ile Asn Thr Ser Thr
         45                  50                  55

GGA GAG TCA ACA TTT GCT GAT GAC TTC AAG GGA CGG TTT GAC   295
Gly Glu Ser Thr Phe Ala Asp Asp Phe Lys Gly Arg Phe Asp
             60                  65                  70

TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT TTG CAG ATC   337
Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala Tyr Leu Gln Ile
                 75                  80

AAC AAC CTC AAA AGT GAA GAC ATG GCT ACA TAT TTC TGT GCA   379
Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr Phe Cys Ala
 85                  90                  95

AGA TGG GAG GTT TAC CAC GGC TAC GTT CCT TAC TGG GGC CAA   421
Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr Trp Gly Gln
    100                 105                 110

GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT GGC GGT TCT GGT   463
Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly
        115                 120                 125

GGC GGT GGC TCC GGC GGT GGC GGT TCT GAC ATC CAG CTG ACC   505
Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr
            130                 135                 140

CAG TCT CAC AAA TTC CTG TCC ACT TCA GTA GGA GAC AGG GTC   547
Gln Ser His Lys Phe Leu Ser Thr Ser Val Gly Asp Arg Val
                145                 150

AGC ATC ACC TGC AAG GCC AGT CAG GAT GTG TAT AAT GCT GTT   589
Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Tyr Asn Ala Val
155                 160                 165
```

```
GCC TGG TAT CAA CAG AAA CCA GGA CAA TCT CCT AAA CTT CTG   631
Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu
    170                 175                 180

ATT TAC TCG GCA TCC TCC CGG TAC ACT GGA GTC CCT TCT CGC   673
Ile Tyr Ser Ala Ser Ser Arg Tyr Thr Gly Val Pro Ser Arg
        185                 190                 195

TTC ACT GGC AGT GGC TCT GGG CCG GAT TTC ACT TTC ACC ATC   715
Phe Thr Gly Ser Gly Ser Gly Pro Asp Phe Thr Phe Thr Ile
            200                 205                 210

AGC AGT GTG CAG GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG   757
Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Phe Cys Gln
                215                 220

CAA CAT TTT CGT ACT CCA TTC ACG TTC GGC TCG GGG ACA AAA   799
Gln His Phe Arg Thr Pro Phe Thr Phe Gly Ser Gly Thr Lys
225                 230                 235

TTG GAG ATC AAA GCT CTA GAG CCT GTT CTG GAA AAC CGG GCT   841
Leu Glu Ile Lys Ala Leu Glu Pro Val Leu Glu Asn Arg Ala
    240                 245                 250

GCT CAG GGC GAT ATT ACT GCA CCC GGC GGT GCT CGC CGT TTA   883
Ala Gln Gly Asp Ile Thr Ala Pro Gly Gly Ala Arg Arg Leu
        255                 260                 265

ACG GGT GAT CAG ACT GCC GCT CTG CGT GAT TCT CTT AGC GAT   925
Thr Gly Asp Gln Thr Ala Ala Leu Arg Asp Ser Leu Ser Asp
            270                 275                 280

AAA CCT GCA AAA AAT ATT ATT TTG CTG ATT GGC GAT GGG ATG   967
Lys Pro Ala Lys Asn Ile Ile Leu Leu Ile Gly Asp Gly Met
                285                 290
```

```
GGG GAC TCG GAA ATT ACT GCC GCA CGT AAT TAT GCC GAA GGT  1009
Gly Asp Ser Glu Ile Thr Ala Ala Arg Asn Tyr Ala Glu Gly
295                 300                 305

GCG GGC GGC TTT TTT AAA GGT ATA GAT GCC TTA CCG CTT ACC  1051
Ala Gly Gly Phe Phe Lys Gly Ile Asp Ala Leu Pro Leu Thr
    310                 315                 320

GGG CAA TAC ACT CAC TAT GCG CTG AAT AAA AAA ACC GGC AAA  1093
Gly Gln Tyr Thr His Tyr Ala Leu Asn Lys Lys Thr Gly Lys
        325                 330                 335

CCG GAC TAC GTC ACC GAC TCG GCT GCA TCA GCA ACC GCC TGG  1135
Pro Asp Tyr Val Thr Asp Ser Ala Ala Ser Ala Thr Ala Trp
            340                 345                 350

TCA ACC GGT GTC AAA ACC TAT AAC GGC GCG CTG GGC GTC GAT  1177
Ser Thr Gly Val Lys Thr Tyr Asn Gly Ala Leu Gly Val Asp
                355                 360

ATT CAC GAA AAA GAT CAC CCA ACG ATT CTG GAA ATG GCA AAA  1219
Ile His Glu Lys Asp His Pro Thr Ile Leu Glu Met Ala Lys
365                 370                 375

GCC GCA GGT CTG GCG ACC GGT AAC GTT TCT ACC GCA GAG TTG  1261
Ala Ala Gly Leu Ala Thr Gly Asn Val Ser Thr Ala Glu Leu
    380                 385                 390

CAG GAT GCC ACG CCC GCT GCG CTG GTG GCA CAT GTG ACC TCG  1303
Gln Asp Ala Thr Pro Ala Ala Leu Val Ala His Val Thr Ser
        395                 400                 405

CGC AAA TGC TAC GGT CCG AGC GCG ACC AGT GAA AAA TGT CCG  1345
Arg Lys Cys Tyr Gly Pro Ser Ala Thr Ser Glu Lys Cys Pro
            410                 415                 420
```

```
GGT AAC GCT CTG GAA AAA GGC GGA AAA GGA TCG ATT ACC GAA  1387
Gly Asn Ala Leu Glu Lys Gly Gly Lys Gly Ser Ile Thr Glu
                425                 430

CAG CTG CTT AAC GCT CGT GCC GAC GTT ACG CTT GGC GGC GGC  1429
Gln Leu Leu Asn Ala Arg Ala Asp Val Thr Leu Gly Gly Gly
435                 440                 445

GCA AAA ACC TTT GCT GAA ACG GCA ACC GCT GGT GAA TGG CAG  1471
Ala Lys Thr Phe Ala Glu Thr Ala Thr Ala Gly Glu Trp Gln
    450                 455                 460

GGA AAA ACG CTG CGT GAA CAG GCA CAG GCG CGT GGT TAT CAG  1513
Gly Lys Thr Leu Arg Glu Gln Ala Gln Ala Arg Gly Tyr Gln
        465                 470                 475

TTG GTG AGC GAT GCT GCC TCA CTG AAT TCG GTG ACG GAA GCG  1555
Leu Val Ser Asp Ala Ala Ser Leu Asn Ser Val Thr Glu Ala
            480                 485                 490

AAT CAG CAA AAA CCC CTG CTT GGC CTG TTT GCT GAC GGC AAT  1597
Asn Gln Gln Lys Pro Leu Leu Gly Leu Phe Ala Asp Gly Asn
                495                 500

ATG CCA GTG CGC TGG CTA GGA CCG AAA GCA ACG TAC CAT GGC  1639
Met Pro Val Arg Trp Leu Gly Pro Lys Ala Thr Tyr His Gly
505                 510                 515

AAT ATC GAT AAG CCC GCA GTC ACC TGT ACG CCA AAT CCG CAA  1681
Asn Ile Asp Lys Pro Ala Val Thr Cys Thr Pro Asn Pro Gln
    520                 525                 530

CGT AAT GAC AGT GTA CCA ACC CTG GCG CAG ATG ACC GAC AAA  1723
Arg Asn Asp Ser Val Pro Thr Leu Ala Gln Met Thr Asp Lys
        535                 540                 545
```

```
GCC ATT GAA TTG TTG AGT AAA AAT GAG AAA GGC TTT TTC CTG  1765
Ala Ile Glu Leu Leu Ser Lys Asn Glu Lys Gly Phe Phe Leu
            550                 555                 560

CAA GTT GAA GGT GCG TCA ATC GAT AAA CAG GAT CAT GCT GCG  1807
Gln Val Glu Gly Ala Ser Ile Asp Lys Gln Asp His Ala Ala
                565                 570

AAT CCT TGT GGG CAA ATT GGC GAG ACG GTC GAT CTC GAT GAA  1849
Asn Pro Cys Gly Gln Ile Gly Glu Thr Val Asp Leu Asp Glu
575                 580                 585

GCC GTA CAA CGG GCG CTG GAA TTC GCT AAA AAG GAG GGT AAC  1891
Ala Val Gln Arg Ala Leu Glu Phe Ala Lys Lys Glu Gly Asn
    590                 595                 600

ACG CTG GTC ATA GTC ACC GCT GAT CAC GCC CAC GCC AGC CAG  1933
Thr Leu Val Ile Val Thr Ala Asp His Ala His Ala Ser Gln
        605                 610                 615

ATT GTT GCG CCG GAT ACC AAA GCT CCG GGC CTC ACC CAG GCG  1975
Ile Val Ala Pro Asp Thr Lys Ala Pro Gly Leu Thr Gln Ala
            620                 625                 630

CTA AAT ACC AAA GAT GGC GCA GTG ATG GTG ATG AGT TAC GGG  2017
Leu Asn Thr Lys Asp Gly Ala Val Met Val Met Ser Tyr Gly
                635                 640

AAC TCC GAA GAG GAT TCA CAA GAA CAT ACC GGC AGT CAG TTG  2059
Asn Ser Glu Glu Asp Ser Gln Glu His Thr Gly Ser Gln Leu
645                 650                 655

CGT ATT GCG GCG TAT GGC CCG CAT GCC GCC AAT GTT GTT GGA  2101
Arg Ile Ala Ala Tyr Gly Pro His Ala Ala Asn Val Val Gly
    660                 665                 670
```

```
CTG ACC GAC CAG ACC GAT CTC TTC TAC ACC ATG AAA GCC GCT  2143
Leu Thr Asp Gln Thr Asp Leu Phe Tyr Thr Met Lys Ala Ala
        675                 680                 685

CTG GGG CTG AAA TAAAACCGCG CCCGGCAGTG AATTTTCGCT        2185
Leu Gly Leu Lys
            690
GCCGGGTGGT TTTTTTGCTG TTAGCAACCA GACTTAATGG CAGAGCTC    2233
```

SEQ ID NO:6

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 342 bp
MOLECULE TYPE: plasmid DNA
ORIGINAL SOURCE ORGANISM: mouse
IMMEDIATE EXPERIMENTAL SOURCE: E.coli
NAME OF CELL CLONE: pWW15-VH51-I

FEATURES:	from 1 to 14 bp	partial sequence of VH1BACK primer region
	from 82 to 96 bp	CDR_{1H}
	from 139 to 189 bp	CDR_{2H}
	from 286 to 318 bp	CDR_{3H}
	from 317 to 342 bp	partial sequence of VH1FOR primer region

PROPERTIES: encodes the heavy chain variable domain of monoclonal antibody FWP51

```
CTGCAGCAGT CTGGGGCTGA GCTGGTGAGG CCTGGGACTT CAGTGAAGCT   50

GTCCTGCAAG GCTTCTGATT ACACCTTCAC CAGCTACTGG ATGAACTGGG  100

TGAAGCAGAG GCCTGGACAA GGCCTTGAAT GGATTGGTAT GATTGATCCT  150

TCAGACAGTG AAACTCAATA CAATCAAATG TTCAAGGACA AGGCCGCATT  200

GACTGTAGAC AAGTCCTCCA ATACAGCCTA CATGCAACTC AGCAGCCTGA  250

CATCTGAGGA CTCTGCGGTC TATTACTGTG CAAAAGGGGG GGCCTCTGGG  300

GACTGGTACT TCGATGTCTG GGGCCAAGGG ACCACGGTCA CC          342
```

SEQ ID NO:7

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 310 bp
MOLECULE TYPE: plasmid DNA
ORIGINAL SOURCE ORGANISM: mouse
IMMEDIATE EXPERIMENTAL SOURCE: E.coli
NAME OF CELL CLONE: pWW15-VL51-1

FEATURES:		
	from 1 to 18 bp	partial sequence of VK1BACK primer region
	from 64 to 96 bp	CDR_{1L}
	from 142 to 162 bp	CDR_{2L}
	from 259 to 282 bp	CDR_{3L}
	from 292 to 310 bp	partial sequence of VK1FOR primer region

PROPERTIES: encodes the light chain variable domain of monoclonal antibody FWP51

```
CAGCTGACCC AGTCTCCATC CTCACTGTCT GCATCTCTGG GAGGCGAAGT     50

CACCATCACT TGCAAGGCAA GCCAAGACAT TAAGAAGTAT ATAGCTTGGT    100

ACCAACACAA GCCTGGAAAA AGTCCTCGGC TACTCATACA CTACACATCT    150

GTATTACAGC CAGGCATCCC ATCCAGGTTC AGTGGAAGTG GGTCTGGGAG    200

AGATTATTCC TTCAGCATCC ACAACCTGGA GCCTGAAGAT ATTGCAACTT    250

ATTATTGTCT ACATTATGAT TATCTGTACA CGTTCGGAGG GGGCACCAAG    300

CTGGAGATCT                                                310
```

SEQ ID NO:8

SEQUENCE TYPE: nucleotide with corresponding protein
SEQUENCE LENGTH: 748 bp
MOLECULE TYPE: plasmid DNA ORIGINAL
SOURCE ORGANISM: mouse
IMMEDIATE EXPERIMENTAL SOURCE: E.coli
NAME OF CELL CLONE: pWW15-Fv51

FEATURES:	from 1 to 8 bp	synthetic spacer
	from 9 to 368 bp	FWP 51 heavy chain variable domain
	from 99 to 113 bp	CDR1H
	from 156 to 206 bp	CDR2H
	from 303 to 335 bp	CDR3H
	from 369 to 413 bp	synthetic spacer
	from 414 to 728 bp	FWP 51 light chain variable domain
	from 483 to 515 bp	CDR1L
	from 561 to 581 bp	R2L
	from 678 to 701 bp	CDR3L
	from 729 to 748 bp	synthetic spacer

PROPERTIES: encodes single-chain Fv fusion gene comprising monoclonal antibody FWP51 heavy and kappa light chain variable domain cDNA

```
AAGCT                                                              5

TCT CAG GTA CAA CTG CAG CAG TCT GGG GCT GAG CTG GTG               44
Ser Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val
 1               5                  10

AGG CCT GGG ACT TCA GTG AAG CTG TCC TGC AAG GCT TCT GAT           86
Arg Pro Gly Thr Ser Val Lys Leu Ser Cys Lys Ala Ser Asp
     15                  20                  25
```

```
TAC ACC TTC ACC AGC TAC TGG ATG AAC TGG GTG AAG CAG AGG  128
Tyr Thr Phe Thr Ser Tyr Trp Met Asn Trp Val Lys Gln Arg
          30                  35                  40

CCT GGA CAA GGC CTT GAA TGG ATT GGT ATG ATT GAT CCT TCA  170
Pro Gly Gln Gly Leu Glu Trp Ile Gly Met Ile Asp Pro Ser
              45                  50                  55

GAC AGT GAA ACT CAA TAC AAT CAA ATG TTC AAG GAC AAG GCC  212
Asp Ser Glu Thr Gln Tyr Asn Gln Met Phe Lys Asp Lys Ala
                  60                  65

GCA TTG ACT GTA GAC AAG TCC TCC AAT ACA GCC TAC ATG CAA  254
Ala Leu Thr Val Asp Lys Ser Ser Asn Thr Ala Tyr Met Gln
 70                  75                  80

CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT TAC TGT  296
Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
      85                  90                  95

GCA AAA GGG GGG GCC TCT GGG GAC TGG TAC TTC GAT GTC TGG  338
Ala Lys Gly Gly Ala Ser Gly Asp Trp Tyr Phe Asp Val Trp
          100                 105                 110

GGC CAA GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT GGC GGT  380
Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly
              115                 120                 125

TCT GGT GGC GGT GGC TCC GGC GGT GGC GGT TCT GAC ATC CAG  422
Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln
                  130                 135

CTG ACC CAG TCT CCA TCC TCA CTG TCT GCA TCT CTG GGA GGC  464
Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Leu Gly Gly
                  145                 150
```

```
GAA GTC ACC ATC ACT TGC AAG GCA AGC CAA GAC ATT AAG AAG  506
Glu Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Lys  168
    155                 160                 165

TAT ATA GCT TGG TAC CAA CAC AAG CCT GGA AAA AGT CCT CGG  548
Tyr Ile Ala Trp Tyr Gln His Lys Pro Gly Lys Ser Pro Arg  182
        170                 175                 180

CTA CTC ATA CAC TAC ACA TCT GTA TTA CAG CCA GGC ATC CCA  590
Leu Leu Ile His Tyr Thr Ser Val Leu Gln Pro Gly Ile Pro
            185                 190                 195

TCC AGG TTC AGT GGA AGT GGG TCT GGG AGA GAT TAT TCC TTC  632
Ser Arg Phe Ser Gly Ser Gly Ser Gly Arg Asp Tyr Ser Phe
                200                 205

AGC ATC CAC AAC CTG GAG CCT GAA GAT ATT GCA ACT TAT TAT  674
Ser Ile His Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr
210                 215                 220

TGT CTA CAT TAT GAT TAT CTG TAC ACG TTC GGA GGG GGC ACC  716
Cys Leu His Tyr Asp Tyr Leu Tyr Thr Phe Gly Gly Gly Thr
    225                 230                 235

AAG CTG GAG ATC TAGCTGATCA AAGCTCTAGA                    748
Lys Leu Glu Ile
        240
```

SEQ ID NO:9

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 201 bp
MOLECULE TYPE: plasmid DNA
ORIGINAL SOURCE ORGANISM: Pseudomonas aeruginosa PAK
IMMEDIATE EXPERIMENTAL SOURCE: E.coli
NAME OF CELL CLONE: pWW22

FEATURES:		
	from 1 to 27 bp	synthetic spacer
	from 29 to 201 bp	partial exotoxin A sequence corresponding to nucleotide positions 1574 to 1747 bp of the exotoxin A sequence (Gray et al., Proc. Natl. Acad. Sci. USA 81: 2645, 1984)

PROPERTIES: encodes part of the mutated exotoxin A gene from Pseudomonas aeruginosa PAK

```
AAGCTTAAGG AGATCTGCAT GCTTCTAGAG GGCGGCAGCC TGGCCGCGCT     50

GACCGCGCAC CAGGCCTGCC ACCTGCCGCT GGAGACTTTC ACCCGTCATC    100

GCCAGCCGCG CGGCTGGGAA CAACTGGAGC AGTGCGGCTA TCCGGTGCAG    150

CGGCTGGTCG CCCTCTACCT GGCGGCGCGA CTGTCATGGA ACCAGGTCGA    200

C                                                         201
```

SEQ ID NO:10

SEQUENCE TYPE: nucleotide with corresponding protein
SEQUENCE LENGTH: 2012 bp
MOLECULE TYPE: plasmid DNA
ORIGINAL SOURCE ORGANISM: mouse/P.aeruginosa
IMMEDIATE EXPERIMENTAL SOURCE: E.coli
NAME OF CELL CLONE: pWW215-5

FEATURES:		
	from 1 to 63 bp	ompA signal peptide
	from 64 to 87 bp	FLAG peptide and enterokinase cleavage site
	from 97 to 453 bp	FRP5 heavy chain variable domain
	from 454 to 498 bp	15 amino acids linker sequence
	from 499 to 822 bp	FRP5 light chain variable domain
	from 826 to 1911 bp	exotoxin A gene coding region (coding for amino acids 252 to 613 of the mature exotoxin A)
	from 1912 to 2012 bp	3'non-coding region of the exotoxin A gene

PROPERTIES: Fv heavy chain/light chain variable domain and exotoxin A fusion protein Fv(FRP5)-ETA binding to the c-erbB-2 protein

```
ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT    42
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly
    -30                 -25                 -20

TTC GCT ACC GTT GCG CAA GCT GAC TAC AAG GAC GAC GAT GAC    84
Phe Ala Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp
        -15                 -10                 -5

AAG CTA GCT TCT CAG GTA CAA CTG CAG CAG TCT GGA CCT GAA   126
Lys Leu Ala Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu
            1               5                  10
```

```
CTG AAG AAG CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GCC  168
Leu Lys Lys Pro Gly Glu Thr Val Lys Ile Ser Cys Lys Ala
                15                  20                  25

TCT GGG TAT CCT TTC ACA AAC TAT GGA ATG AAC TGG GTG AAG  210
Ser Gly Tyr Pro Phe Thr Asn Tyr Gly Met Asn Trp Val Lys
                30                  35

CAG GCT CCA GGA CAG GGT TTA AAG TGG ATG GGC TGG ATT AAC  252
Gln Ala Pro Gly Gln Gly Leu Lys Trp Met Gly Trp Ile Asn
 40                  45                  50

ACC TCC ACT GGA GAG TCA ACA TTT GCT GAT GAC TTC AAG GGA  294
Thr Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp Phe Lys Gly
     55                  60                  65

CGG TTT GAC TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT  336
Arg Phe Asp Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala Tyr
         70                  75                  80

TTG CAG ATC AAC AAC CTC AAA AGT GAA GAC ATG GCT ACA TAT  378
Leu Gln Ile Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr
                85                  90                  95

TTC TGT GCA AGA TGG GAG GTT TAC CAC GGC TAC GTT CCT TAC  420
Phe Cys Ala Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr
            100                 105

TGG GGC CAA GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT GGC  462
Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly
110                 115                 120

GGT TCT GGT GGC GGT GGC TCC GGC GGT GGC GGT TCT GAC ATC  504
Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile
    125                 130                 135
```

```
CAG CTG ACC CAG TCT CAC AAA TTC CTG TCC ACT TCA GTA GGA  546
Gln Leu Thr Gln Ser His Lys Phe Leu Ser Thr Ser Val Gly
        140                 145                 150

GAC AGG GTC AGC ATC ACC TGC AAG GCC AGT CAG GAT GTG TAT  588
Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Tyr
            155                 160                 165

AAT GCT GTT GCC TGG TAT CAA CAG AAA CCA GGA CAA TCT CCT  630
Asn Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro
            170                 175

AAA CTT CTG ATT TAC TCG GCA TCC TCC CGG TAC ACT GGA GTC  672
Lys Leu Leu Ile Tyr Ser Ala Ser Ser Arg Tyr Thr Gly Val
180                 185                 190

CCT TCT CGC TTC ACT GGC AGT GGC TCT GGG CCG GAT TTC ACT  714
Pro Ser Arg Phe Thr Gly Ser Gly Ser Gly Pro Asp Phe Thr
    195                 200                 205

TTC ACC ATC AGC AGT GTG CAG GCT GAA GAC CTG GCA GTT TAT  756
Phe Thr Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr
        210                 215                 220

TTC TGT CAG CAA CAT TTT CGT ACT CCA TTC ACG TTC GGC TCG  798
Phe Cys Gln Gln His Phe Arg Thr Pro Phe Thr Phe Gly Ser
            225                 230                 235

GGG ACA AAA TTG GAG ATC AAA GCT CTA GAG GGC GGC AGC CTG  840
Gly Thr Lys Leu Glu Ile Lys Ala Leu Glu Gly Gly Ser Leu
            240                 245

GCC GCG CTG ACC GCG CAC CAG GCC TGC CAC CTG CCG CTG GAG  882
Ala Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu
250                 255                 260
```

```
ACT TTC ACC CGT CAT CGC CAG CCG CGC GGC TGG GAA CAA CTG  924
Thr Phe Thr Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu
    265                 270                 275

GAG CAG TGC GGC TAT CCG GTG CAG CGG CTG GTC GCC CTC TAC  966
Glu Gln Cys Gly Tyr Pro Val Gln Arg Leu Val Ala Leu Tyr
        280                 285                 290

CTG GCG GCG CGA CTG TCA TGG AAC CAG GTC GAC CAG GTG ATC 1008
Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp Gln Val Ile
            295                 300                 305

CGC AAC GCC CTG GCC AGC CCC GGC AGC GGC GGC GAC CTG GGC 1050
Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly
                310                 315

GAA GCG ATC CGC GAG CAG CCG GAG CAG GCC CGT CTG GCC CTG 1092
Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu Ala Leu
320                 325                 330

ACC CTG GCC GCC GCC GAG AGC GAG CGC TTC GTC CGG CAG GGC 1134
Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln Gly
    335                 340                 345

ACC GGC AAC GAC GAG GCC GGC GCG GCC AAC GCC GAC GTG GTG 1176
Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val
        350                 355                 360

AGC CTG ACC TGC CCG GTC GCC GCC GGT GAA TGC GCG GGC CCG 1218
Ser Leu Thr Cys Pro Val Ala Ala Gly Glu Cys Ala Gly Pro
            365                 370                 375

GCG GAC AGC GGC GAC GCC CTG CTG GAG CGC AAC TAT CCC ACT 1260
Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr
                380                 385
```

```
GGC GCG GAG TTC CTC GGC GAC GGC GGC GAC GTC AGC TTC AGC 1302
Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe Ser
390                 395                 400

ACC CGC GGC ACG CAG AAC TGG ACG GTG GAG CGG CTG CTC CAG 1344
Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln
    405                 410                 415

GCG CAC CGC CAA CTG GAG GAG CGC GGC TAT GTG TTC GTC GGC 1386
Ala His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly
        420                 425                 430

TAC CAC GGC ACC TTC CTC GAA GCG GCG CAA AGC ATC GTC TTC 1428
Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe
            435                 440                 445

GGC GGG GTG CGC GCG CGC AGC CAG GAC CTC GAC GCG ATC TGG 1470
Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp
                450                 455

CGC GGT TTC TAT ATC GCC GGC GAT CCG GCG CTG GCC TAC GGC 1512
Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly
460                 465                 470

TAC GCC CAG GAC CAG GAA CCC GAC GCA CGC GGC CGG ATC CGC 1554
Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg
    475                 480                 485

AAC GGT GCC CTG CTG CGG GTC TAT GTG CCG CGC TCG AGC CTG 1596
Asn Gly Ala Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu
        490                 495                 500

CCG GGC TTC TAC CGC ACC AGC CTG ACC CTG GCC GCG CCG GAG 1638
Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala Pro Glu
            505                 510                 515
```

```
GCG GCG GGC GAG GTC GAA CGG CTG ATC GGC CAT CCG CTG CCG 1680
Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro
                520                 525

CTG CGC CTG GAC GCC ATC ACC GGC CCC GAG GAG GAA GGC GGG 1722
Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly Gly
530                 535                 540

CGC CTG GAG ACC ATT CTC GGC TGG CCG CTG GCC GAG CGC ACC 1764
Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr
    545                 550                 555

GTG GTG ATT CCC TCG GCG ATC CCC ACC GAC CCG CGC AAC GTC 1806
Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val
        560                 565                 570

GGC GGC GAC CTC GAC CCG TCC AGC ATC CCC GAC AAG GAA CAG 1848
Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln
            575                 580                 585

GCG ATC AGC GCC CTG CCG GAC TAC GCC AGC CAG CCC GGC AAA 1890
Ala Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys
                590                 595

CCG CCG CGC GAG GAC CTG AAG TAA CTGCCGCGAC CGGCCGGCTC   1934
Pro Pro Arg Glu Asp Leu Lys
600                 605

CCTTCGCAGG AGCCGGCCTT CTCGGGGCCT GGCCATACAT CAGGTTTTCC  1984

TGATGCCAGC CCAATCGAAT ATGAATTC                          2012
```

SEQ ID NO:11

SEQUENCE TYPE: nucleotide with corresponding protein
SEQUENCE LENGTH: 2012 bp
MOLECULE TYPE: plasmid DNA
ORIGINAL SOURCE ORGANISM: mouse/P.aeruginosa
IMMEDIATE EXPERIMENTAL SOURCE: E.coli
NAME OF CELL CLONE: pWW215-51

FEATURES:		
	from 1 to 63 bp	ompA signal peptide
	from 64 to 87 bp	FLAG peptide and enterokinase cleavage site
	from 97 to 456 bp	FWP51 heavy chain variable domain
	from 457 to 501 bp	15 amino acids linker sequence
	from 502 to 822 bp	FWP51 light chain variable domain
	from 826 to 1911 bp	exotoxin A gene coding region (coding for amino acids 252 to 613 of the mature exotoxin A)
	from 1912 to 2012 bp	3' non-coding region of the exotoxin A gene

PROPERTIES: Fv heavy chain/light chain variable domain and exotoxin A fusion protein Fv(FWP51)-ETA binding to the c-erbB-2 protein

```
ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT   42
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly
    -30                 -25                 -20

TTC GCT ACC GTT GCG CAA GCT GAC TAC AAG GAC GAC GAT GAC   84
Phe Ala Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp
        -15                 -10                  -5

AAG CTA GCT TCT CAG GTA CAA CTG CAG CAG TCT GGG GCT GAG  126
Lys Leu Ala Ser Gln Val Gln Leu Gln Gln Ser Gly Ala Glu
            1                   5                  10
```

```
CTG GTG AGG CCT GGG ACT TCA GTG AAG CTG TCC TGC AAG GCT  168
Leu Val Arg Pro Gly Thr Ser Val Lys Leu Ser Cys Lys Ala
                15                  20                  25

TCT GAT TAC ACC TTC ACC AGC TAC TGG ATG AAC TGG GTG AAG  210
Ser Asp Tyr Thr Phe Thr Ser Tyr Trp Met Asn Trp Val Lys
                30                  35

CAG AGG CCT GGA CAA GGC CTT GAA TGG ATT GGT ATG ATT GAT  252
Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile Gly Met Ile Asp
 40                  45                  50

CCT TCA GAC AGT GAA ACT CAA TAC AAT CAA ATG TTC AAG GAC  294
Pro Ser Asp Ser Glu Thr Gln Tyr Asn Gln Met Phe Lys Asp
     55                  60                  65

AAG GCC GCA TTG ACT GTA GAC AAG TCC TCC AAT ACA GCC TAC  336
Lys Ala Ala Leu Thr Val Asp Lys Ser Ser Asn Thr Ala Tyr
         70                  75                  80

ATG CAA CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT  378
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
                85                  90                  95

TAC TGT GCA AAA GGG GGG GCC TCT GGG GAC TGG TAC TTC GAT  420
Tyr Cys Ala Lys Gly Gly Ala Ser Gly Asp Trp Tyr Phe Asp
                100                 105

GTC TGG GGC CAA GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT  462
Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly
110                 115                 120

GGC GGT TCT GGT GGC GGT GGC TCC GGC GGT GGC GGT TCT GAC  504
Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp
    125                 130                 135
```

```
ATC CAG CTG ACC CAG TCT CCA TCC TCA CTG TCT GCA TCT CTG  546
Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Leu
        140                 145                 150

GGA GGC GAA GTC ACC ATC ACT TGC AAG GCA AGC CAA GAC ATT  588
Gly Gly Glu Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile
            155                 160                 165

AAG AAG TAT ATA GCT TGG TAC CAA CAC AAG CCT GGA AAA AGT  630
Lys Lys Tyr Ile Ala Trp Tyr Gln His Lys Pro Gly Lys Ser
                170                 175

CCT CGG CTA CTC ATA CAC TAC ACA TCT GTA TTA CAG CCA GGC  672
Pro Arg Leu Leu Ile His Tyr Thr Ser Val Leu Gln Pro Gly
180                 185                 190

ATC CCA TCC AGG TTC AGT GGA AGT GGG TCT GGG AGA GAT TAT  714
Ile Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Arg Asp Tyr
    195                 200                 205

TCC TTC AGC ATC CAC AAC CTG GAG CCT GAA GAT ATT GCA ACT  756
Ser Phe Ser Ile His Asn Leu Glu Pro Glu Asp Ile Ala Thr
        210                 215                 220

TAT TAT TGT CTA CAT TAT GAT TAT CTG TAC ACG TTC GGA GGG  798
Tyr Tyr Cys Leu His Tyr Asp Tyr Leu Tyr Thr Phe Gly Gly
            225                 230                 235

GGC ACC AAG CTG GAG ATC AAA GCT CTA GAG GGC GGC AGC CTG  840
Gly Thr Lys Leu Glu Ile Lys Ala Leu Glu Gly Gly Ser Leu
                240                 245

GCC GCG CTG ACC GCG CAC CAG GCC TGC CAC CTG CCG CTG GAG  882
Ala Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu
250                 255                 260
```

```
ACT TTC ACC CGT CAT CGC CAG CCG CGC GGC TGG GAA CAA CTG  924
Thr Phe Thr Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu
    265                 270                 275

GAG CAG TGC GGC TAT CCG GTG CAG CGG CTG GTC GCC CTC TAC  966
Glu Gln Cys Gly Tyr Pro Val Gln Arg Leu Val Ala Leu Tyr
        280                 285                 290

CTG GCG GCG CGA CTG TCA TGG AAC CAG GTC GAC CAG GTG ATC 1008
Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp Gln Val Ile
            295                 300                 305

CGC AAC GCC CTG GCC AGC CCC GGC AGC GGC GGC GAC CTG GGC 1050
Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly
                310                 315

GAA GCG ATC CGC GAG CAG CCG GAG CAG GCC CGT CTG GCC CTG 1092
Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu Ala Leu
320                 325                 330

ACC CTG GCC GCC GCC GAG AGC GAG CGC TTC GTC CGG CAG GGC 1134
Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln Gly
    335                 340                 345

ACC GGC AAC GAC GAG GCC GGC GCG GCC AAC GCC GAC GTG GTG 1176
Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val
        350                 355                 360

AGC CTG ACC TGC CCG GTC GCC GCC GGT GAA TGC GCG GGC CCG 1218
Ser Leu Thr Cys Pro Val Ala Ala Gly Glu Cys Ala Gly Pro
            365                 370                 375

GCG GAC AGC GGC GAC GCC CTG CTG GAG CGC AAC TAT CCC ACT 1260
Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr
                380                 385
```

```
GGC GCG GAG TTC CTC GGC GAC GGC GGC GAC GTC AGC TTC AGC 1302
Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe Ser
390                 395                 400

ACC CGC GGC ACG CAG AAC TGG ACG GTG GAG CGG CTG CTC CAG 1344
Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln 448
    405                 410                 415

GCG CAC CGC CAA CTG GAG GAG CGC GGC TAT GTG TTC GTC GGC 1386
Ala His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly
        420                 425                 430

TAC CAC GGC ACC TTC CTC GAA GCG GCG CAA AGC ATC GTC TTC 1428
Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe
            435                 440                 445

GGC GGG GTG CGC GCG CGC AGC CAG GAC CTC GAC GCG ATC TGG 1470
Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp
                450                 455

CGC GGT TTC TAT ATC GCC GGC GAT CCG GCG CTG GCC TAC GGC 1512
Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly
460                 465                 470

TAC GCC CAG GAC CAG GAA CCC GAC GCA CGC GGC CGG ATC CGC 1554
Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg
    475                 480                 485

AAC GGT GCC CTG CTG CGG GTC TAT GTG CCG CGC TCG AGC CTG 1596
Asn Gly Ala Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu
        490                 495                 500

CCG GGC TTC TAC CGC ACC AGC CTG ACC CTG GCC GCG CCG GAG 1638
Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala Pro Glu
            505                 510                 515
```

```
GCG GCG GGC GAG GTC GAA CGG CTG ATC GGC CAT CCG CTG CCG 1680
Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro
                520                 525

CTG CGC CTG GAC GCC ATC ACC GGC CCC GAG GAG GAA GGC GGG 1722
Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly Gly
530                 535                 540

CGC CTG GAG ACC ATT CTC GGC TGG CCG CTG GCC GAG CGC ACC 1764
Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr
    545                 550                 555

GTG GTG ATT CCC TCG GCG ATC CCC ACC GAC CCG CGC AAC GTC 1806
Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val
        560                 565                 570

GGC GGC GAC CTC GAC CCG TCC AGC ATC CCC GAC AAG GAA CAG 1848
Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln
            575                 580                 585

GCG ATC AGC GCC CTG CCG GAC TAC GCC AGC CAG CCC GGC AAA 1890
Ala Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys
                590                 595

CCG CCG CGC GAG GAC CTG AAG TAA  CTGCCGCGAC CGGCCGGCTC  1934
Pro Pro Arg Glu Asp Leu Lys
600                 605

CCTTCGCAGG AGCCGGCCTT CTCGGGGCCT GGCCATACAT CAGGTTTTCC  1984

TGATGCCAGC CCAATCGAAT ATGAATTC                          2012
```

Claims

1. A recombinant antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 comprising a heavy chain variable domain and a light chain variable domain of a monoclonal antibody.

2. A recombinant antibody according to claim 1 wherein the heavy chain variable domain comprises a polypeptide of the formula

$$FR_1\text{-}CDR_{1H}\text{-}FR_2\text{-}CDR_{2H}\text{-}FR_3\text{-}CDR_{3H}\text{-}FR_4 \qquad (I)$$

wherein FR_1 is a polypeptide residue comprising 25-33 naturally occurring amino acids, FR_2 is a polypep-

tide residue comprising 12-16 naturally occurring amino acids, FR_3 is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR_4 is a polypeptide residue comprising 6-13 naturally occurring amino acids, CDR_{1H} is a polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:4, CDR_{2H} is a polypeptide residue of the amino acid sequence 51 to 67 of SEQ ID NO:4, and CDR_{3H} is a polypeptide residue of the amino acid sequence 100 to 109 of SEQ ID NO:4, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

3. A recombinant antibody according to claim 2 wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:4, wherein optionally one or more single amino acids within the amino acid sequences 2 to 31 (FR_1), 37 to 50 (FR_2), 68 to 99 (FR_3), and/or 110 to 120 (FR_4) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

4. A recombinant antibody according to claim 2 wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:4, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

5. A recombinant antibody according to claim 1 wherein the light chain vlable domain comprises a polypeptide of the formula

$$FR_6\text{-}CDR_{1L}\text{-}FR_7\text{-}CDR_{2L}\text{-}FR_8\text{-}CDR_{3L}\text{-}FR_9 \qquad (II)$$

wherein FR_6 is a polypeptide residue comprising naturally occurring amino acids, FR_7 is e polypeptide residue comprising 13-17 naturally occurring amino acids, FR_8 is a polypeptide residue comprising 30-34 naturally occurring amino ecids, FR_9 is a polypeptide residue comprising naturally occurring amino acids, CDR_{1L} is e polypeptide residue of the amino acid sequence 159 to 169 of SEQ ID NO:4, CDR_{2L} is a polypeptide residue of the amino ecid sequence 185 to 191 of SEQ ID NO:4, and CDR_{3L} is a polypeptide residue of the amino acid sequence 224 to 232 of SEQ ID NO:4, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

6. A recombinant antibody according to claim 5 wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:4, wherein optionally one or more single amino acids within the amino acid sequences 136 to 158 (FR_6), 170 to 184 (FR_7), 192 to 223 (FR_8), and/or 233 to 241 (FR_9) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

7. A recombinant antibody according to claim 5 wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:4, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

8. A recombinant antibody according to claim 1 wherein the heavy chain varieble domain comprises a polypeptide of the formula

$$FR_1\text{-}CDR_{1H}\text{-}FR_2\text{-}CDR_{2H}\text{-}FR_3\text{-}CDR_{3H}\text{-}FR_4 \qquad (I)$$

wherein FR_1 is a polypeptide residue comprising 25-33 naturally occurring amino acids, FR_2 is a polypeptide residue comprising 12-16 naturally occurring amino acids, FR_3 is a polypeptide residue comprising 30-34 naturally occurring amino ecids, FR_4 is a polypeptide residue comprising 6-13 naturally occurring amino acids, CDR_{1H} is a polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:8, CDR_{2H} is a polypeptide residue of the emino acid sequence 51 to 67 of SEQ ID NO: 8, and CDR_{3H} is a polypeptide residue of the amino acid sequence 100 to 110 of SEQ ID NO:8, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

9. A recombinant antibody according to claim 8 wherein the heavy chain varieble domain comprises a polypeptide of the amino acid sequence 2 to 121 of SEQ ID NO:8, wherein optionally one or more single amino ecids within the amino acid sequences 2 to 31 (FR_1), 37 to 50 (FR_2), 68 to 99 (FR_3), and/or 111 to 121 (FR_4) are replaced by other amino acids or deleted, end wherein the amino acid Cys mey be in the oxidized state forming S-S-bridges.

10. A recombinant antibody according to claim 8 wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 121 of SEQ ID NO:8, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

11. A recombinant antibody according to claim 1 wherein the light chain variable domain comprises a polypeptide of the formula

$$FR_6\text{-}CDR_{1L}\text{-}FR_7\text{-}CDR_{2L}\text{-}FR_8\text{-}CDR_{3L}\text{-}FR_9 \qquad (II)$$

wherein FR_6 is a polypeptide residue comprising naturally occurring amino acids, FR_7 is a polypeptide residue comprising 13-17 naturally occurring amino acids, FR_8 is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR_9 is a polypeptide residue comprising naturally occurring amino acids, CDR_{1L} is a polypeptide residue of the amino acid sequence 160 to 170 of SEQ ID NO:8, CDR_{2L} is a polypeptide residue of the amino acid sequence 186 to 192 of SEQ ID NO: 8, and CDR_{3L} is a polypeptide residue of the amino acid sequence 225 to 232 of SEQ ID NO:8, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

12. A recombinant antibody according to claim 11 wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO: 8, wherein optionally one or more single amino acids within the amino acid sequences 137 to 159 (FR_6), 171 to 185 (FR_7), 193 to 224 (FR_8), and/or 233 to 241 (FR_9) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

13. A recombinant antibody according to claim 11 wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:8, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

14. A recombinant antibody according to claim 1 which is a chimeric antibody consisting of a mouse heavy chain variable domain with the specificity for c-erbB-2 and a human heavy chain constant domain α, γ, δ, ε or μ, and of a mouse light chain variable domain with the specificity for c-erbB-2 and a human light chain constant domain κ or λ, all assembled to give a functional antibody.

15. A recombinant antibody according to claim 1 which is a single-chain antibody wherein the heavy chain variable domain and the light chain variable domain are linked by a polypeptide spacer group.

16. A single-chain recombinant antibody according to claim 15 further comprising an effector molecule and optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer.

17. A single-chain recombinant antibody according to claim 16 wherein the effector molecule is an enzyme or a biologically active variant thereof.

18. A single-chain recombinant antibody according to claim 16 wherein the enzyme is alkaline phosphatase or a biologically active variant thereof.

19. A single-chain recombinant antibody according to claim 16 wherein the effector molecule is a toxin or a biologically active variant thereof.

20. A single-chain recombinant antibody according to claim 19 wherein the effector molecule is Pseudomonas exotoxin or a biologically active variant therof.

21. A single-chain recombinant antibody according to claim 16 wherein the heavy chain variable domain and the light chain variable domain are derived from a mouse monoclonal antibody selected from the group consisting of FRP5, FSP16, FWP51 and FSP77.

22. A single-chain recombinant antibody according to claim 21, further comprising an effector molecule or a biologically active variant thereof.

23. A single-chain recombinant antibody according to claim 21 wherein the heavy chain variable domain and the light chain variable domain are derived from the mouse monoclonal antibody FRP5.

24. A single chain recombinant antibody according to claim 21 wherein the heavy chain variable domain and the light chain variable domain are derived from the mouse monoclonal antibody FWP51.

25. A single-chain recombinant antibody according to claim 22 comprising the heavy chain variable domain of the mouse monoclonal antibody FRP5, the 15 amino acid polypeptide consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser, the light chain variable domain of the mouse monoclonal antibody FRP5, and

an enzyme or a toxin, or a biologically active variant thereof.

26. A single-chain recombinant antibody designated Fv(FRP5)-phoA according to claim 25 comprising a polypeptide of the amino acid sequence 2 to 690 of SEQ ID NO:5.

27. A single-chain recombinant antibody designated Fv(FRP5)-ETA according to claim 25 comprising a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO: 10.

28. A single-chain recombinant antibody according to claim 22 comprising the heavy chain variable domain of the mouse monoclonal antibody FWP51, the 15 amino acid polypeptide consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser, the light chain variable domain of the mouse monoclonal antibody FWP51, and an enzyme or a toxin, or a biologically active variant thereof.

29. A single-chain recombinant antibody designated Fv(FWP51)-ETA according to claim 28 comprising a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO: 11.

30. A mouse monoclonal antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 selected from the group consisting of antibodies designated FRP5, FSP16, FSP77 and FWP51.

31. A monoclonal antibody according to claim 30 designated FRP5.

32. A monoclonal antibody according to claim 30 designated or FWP51.

33. A method of manufacture of a recombinant antibody according to claim 1 or of a monoclonal antibody according to claim 30, characterized in that cells producing such an antibody are multiplied in vitro or in vivo and, when required, the obtained antibody is isolated.

34. A hybridoma cell secreting a monoclonal antibody according to claims 30.

35. A process for the preparation of a hybridoma cell line according to claim 34 secreting monoclonal antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2, characterized in that a suitable mammal is immunized with purified c-erbB-2 protein, an antigenic carrier containing purified c-erbB-2 or with cells bearing c-erbB-2, antibody-producing cells of the immunized mammal are fused with cells of a suitable myeloma cell line, the hybrid cells obtained in the fusion are cloned, and cell clones secreting the desired antibodies are selected.

36. A recombinant DNA comprising an insert coding for a recombinant antibody according to claim 1.

37. A recombinant DNA according to claim 36 comprising an insert coding for a heavy chain murine variable domain of a monoclonal antibody selected from the group consisting of antibodies FRP5, FSP16, FSP77 and FWP51 or coding for an amino acid sequence homologous to said heavy chain variable domain.

38. A recombinant DNA according to claim 36 comprising an insert coding for a light chain murine variable domain of a monoclonal antibody selected from the group consisting of antibodies FRP5, FSP16, FSP77 and FWP51 or coding for an amino acid sequence homologous to said light chain variable domain.

39. A recombinant DNA according to claim 36 which is a hybrid vector further comprising an origin of replication or an autonomously replicating sequence, one or more dominant marker sequences and, optionally, expression control sequences, signal sequences and additional restriction sites.

40. A hybrid vector according to claim 39 comprising a Simian virus promoter and the mouse Ig H or L chain enhancer.

41. A process for the preparation of a DNA according to claim 36 comprising the steps of
a) preparing murine DNA from the genome of a suitable hybridoma cell line and selecting the desired DNA coding for the variable heavy and/or light chain domains of the antibody with the desired specificity,
b) preparing DNA coding for the desired signal sequence and/or preparing DNA coding for an effector molecule,
c) synthesizing DNA coding for the desired spacer group by chemical methods,
d) constructing recombinant genes encoding the recombiant antibodies by incorporating the DNA of

step a) and, optionally, b) end/or c) into appropriate hybrid vectors,
e) transferring the obtained hybrid vectors into a recipient host cell or retrieving the DNA coding for the recombinant genes and transferring the unlinked DNA into a recipient host cell,
f) selecting and culturing the transformed host cell, and
g) optionally isolating the desired DNA.

42. A host cell transformed with a recombinant DNA according to claim 36.

43. A host cell according to claim 42 which is a cell of a strain of E. coli.

44. A process for the preparation of a transformed host cell according to claim 42 wherein suitable recipient cells are transformed with a hybrid vector comprising a DNA insert coding for a heavy chain murine variable domain and/or for a light chain murine variable domain of an antibody directed to the extracellular domain of the growth factor receptor c-erbB-2, an origin of replication or an autonomously replicating sequence, one or more dominant marker sequences and, optionally, expression control sequences, signal sequences and additional restriction sites, and the transformed cells are selected.

45. Use of a recombinant antibody according to claim 1 for the qualitative and quantitative determination of the growth factor receptor c-erbB-2.

46. Use of a monoclonal antibody according to claim 30 for the qualitative and quantitative determination of the growth factor receptor c-erbB-2.

47. Use according to claim 45 comprising immunostaining of tissue sections with a solution containing the recombinant antibody comprising a detectable enzyme.

48. A test kit for the qualitative and quantitative determination of c-erbB-2 protein comprising a recombinant antibody according to claim 1 and/or a monoclonal antibody according to claim 30.

49. A recombinant antibody according to claim 1 for use in the treatment of the human or animal body.

50. A monoclonal antibody according to claim 30 for use in the treatment of the human or animal body.

51. A pharmaceutical composition for treating tumors over-expressing the growth factor receptor c-erbB-2 comprising a therapeutically effective amount of a recombinant antibody according to claim 1 or of a monoclonal antibody according to claim 30 and a pharmaceutically acceptable carrier.

52. The use of a recombinant antibody according to claim 1 and/or a monoclonal antibody according to claim 30 for the manufacture of a pharmaceutical preparation.

Claims for the following Contracting State : ES

1. Process for the preparation of a recombinant antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 comprising a heavy chain variable domain and a light chain variable domain of a monoclonal antibody characterized in that cells producing such an antibody are multiplied in vitro or in vivo and, when required, the obtained antibody is isolated.

2. Process according to claim 1 for the preparation of a recombinant antibody wherein the heavy chain variable domain comprises a polypeptide of the formula

$$FR_1\text{-}CDR_{1H}\text{-}FR_2\text{-}CDR_{2H}\text{-}FR_3\text{-}CDR_{3H}\text{-}FR_4 \qquad (I)$$

wherein FR_1 is a polypeptide residue comprising 25-33 naturally occurring amino acids, FR_2 is a polypeptide residue comprising 12-16 naturally occurring amino acids, FR_3 is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR_4 is a polypeptide residue comprising 6-13 naturally occurring amino acids, CDR_{1H} is a polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:4, CDR_{2H} is a polypeptide residue of the amino acid sequence 51 to 67 of SEQ ID NO:4, and CDR_{3H} is a polypeptide residue of the amino acid sequence 100 to 109 of SEQ ID NO:4, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

3. Process according to claim 2 for the preparation of a recombinant antibody wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:4, wherein

optionally one or more single amino acids within the amino acid sequences 2 to 31 (FR_1), 37 to 50 (FR_2), 68 to 99 (FR_3), and/or 110 to 120 (FR_4) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

4. Process according to claim 2 for the preparation of a recombinant antibody wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:4, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

5. Process according to claim 1 for the preparation of a recombinant antibody wherein the light chain variable domain comprises a polypeptide of the formula

$$FR_6\text{-}CDR_{1L}\text{-}FR_7\text{-}CDR_{2L}\text{-}FR_8\text{-}CDR_{3L}\text{-}FR_9 \qquad (II)$$

wherein FR_6 is a polypeptide residue comprising naturally occurring amino acids, FR_7 is a polypeptide residue comprising 13-17 naturally occurring amino acids, FR_8 is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR_9 is a polypeptide residue comprising naturally occurring amino acids, CDR_{1L} is a polypeptide residue of the amino acid sequence 159 to 169 of SEQ ID NO:4, CDR_{2L} is a polypeptide residue of the amino acid sequence 185 to 191 of SEQ ID NO:4, and CDR_{3L} is a polypeptide residue of the amino acid sequence 224 to 232 of SEQ ID NO:4, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

6. Process according to claim 5 for the preparation of a recombinant antibody wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:4, wherein optionally one or more single amino acids within the amino acid sequences 136 to 158 (FR_6), 170 to 184 (FR_7), 192 to 223 (FR_8), and/or 233 to 241 (FR_9) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

7. Process according to claim 5 for the preparation of a recombinant antibody wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:4, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

8. Process according to claim 1 for the preparation of a recombinant antibody wherein the heavy chain variable domain comprises a polypeptide of the formula

$$FR_1\text{-}CDR_{1H}\text{-}FR_2\text{-}CDR_{2H}\text{-}FR_3\text{-}CDR_{3H}\text{-}FR_4 \qquad (I)$$

wherein FR_1 is a polypeptide residue comprising 25-33 naturally occurring amino acids, FR_2 is a polypeptide residue comprising 12-16 naturally occurring amino acids, FR_3 is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR_4 is a polypeptide residue comprising 6-13 naturally occurring amino acids, CDR_{1H} is a polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:8, CDR_{2H} is a polypeptide residue of the amino acid sequence 51 to 67 of SEQ ID NO:8, and CDR_{3H} is a polypeptide residue of the amino acid sequence 100 to 110 of SEQ ID NO:8, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

9. Process according to claim 8 for the preparation of a recombinant antibody wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 121 of SEQ ID NO:8, wherein optionally one or more single amino acids within the amino acid sequences 2 to 31 (FR_1), 37 to 50 (FR_2), 68 to 99 (FR_3), and/or 111 to 121 (FR_4) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

10. Process according to claim 8 for the preparation of a recombinant antibody wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 121 of SEQ ID NO:8, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

11. Process according to claim 1 for the preparation of a recombinant antibody wherein the light chain variable domain comprises a polypeptide of the formula

$$FR_6\text{-}CDR_{1L}\text{-}FR_7\text{-}CDR_{2L}\text{-}FR_8\text{-}CDR_{3L}\text{-}FR_9 \qquad (II)$$

wherein FR_6 is a polypeptide residue comprising naturally occurring amino acids, FR_7 is a polypeptide residue comprising 13-17 naturally occurring amino acids, FR_8 is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR_9 is a polypeptide residue comprising naturally occurring amino acids, CDR_{1L} is a polypeptide residue of the amino acid sequence 160 to 170 of SEQ ID NO:8, CDR_{2L} is a polypeptide residue of the amino acid sequence 186 to 192 of SEQ ID NO: 8, and CDR_{3L} is a polypeptide residue of the amino acid sequence 225 to 232 of SEQ ID NO:8, and wherein the amino acid Cys may be

in the oxidized state forming S-S-bridges.

12. Process according to claim 11 for the preparation of a recombinant antibody wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:8, wherein optionally one or more single amino acids within the amino acid sequences 137 to 159 (FR_6), 171 to 185 (FR_7), 193 to 224 (FR_8), and/or 233 to 241 (FR_9) are replaced by other amino acids or deletad, and wherein tha amino acid Cys may be in the oxidized state forming S-S-bridges.

13. Process according to claim 11 for the preparation of a recombinant antibody wherein the light chain variabla domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:8, wherein tha amino acid Cys may be in the oxidized state forming S-S-bridges.

14. Process according to claim 1 for the preparation of a recombinant antibody which is a chimeric antibody consisting of a mouse heavy chain variable domain with the specificity for c-erbB-2 and a human haavy chain constant domain α, γ, δ, ε or μ, and of a mouse light chain variable domain with the specificity for c-erbB-2 and a human light chain constant domain κ or λ, all assembled to give a functional antibody.

15. Process according to claim 1 for the preparation of a recombinant antibody which is a single-chain antibody wherein the heavy chain viable domain and the light chain variable domain are linked by a polypeptida spacar group.

16. Process according to claim 15 for the preparation of a single-chain recombinant antibody further comprising an effector molecule and optionally comprising a peptide facilitating purification, a cleavage site and a peptida spacer.

17. Process according to claim 16 for the preparation of a single-chain recombinant antibody wherein the effactor molecule is an enzyme or a biologically active variant thereof.

18. Process according to claim 16 for tha preparation of a single-chain recombinant antibody wherein the enzyme is alkaline phosphatase or a biologically active variant thereof.

19. Process according to claim 16 for the preparation of a singla-chain recombinant antibody wherain the effector molacule is a toxin or a biologically active variant thereof.

20. Process according to claim 19 for the preparation of a single-chain recombinant antibody wherein the effector molecule is Pseudomonas exotoxin or a biologically active variant therof.

21. Process according to claim 19 for tha praparation of a single-chain recombinant antibody wherein the haavy chain variable domain and the light chain variable domain ara derived from a mouse monoclonal antibody selected from the group consisting of FRP5, FSP16, FWP51 and FSP77.

22. Process according to claim 21 for the preparation of a single-chain racombinant antibody, further comprising an effector molecule or a biologically active variant tharaof.

23. Process according to claim 21 for the preparation of a single-chain recombinant antibody wherein the haavy chain variabla domain and tha light chain variabla domain are derived from tha mousa monoclonal antibody FRP5.

24. Process according to claim 21 for the preparation of a single chain recombinant antibody wherein the heavy chain variable domain and the light chain variabla domain ara darivad from tha mouse monoclonal antibody FWP51.

25. Process according to claim 22 for the preparation of a single-chain recombinant antibody comprising the haavy chain variable domain of tha mousa monoclonal antibody FRP5, the 15 amino acid polypaptide consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser, tha light chain variable domain of the mouse monoclonal antibody FRP5, and an anzyma or a toxin, or a biologically active variant thereof.

26. Process according to claim 25 for the preparation of a single-chain recombinant antibody dasignated Fv(FRP5)-phoA comprising a polypeptide of the amino acid sequence 2 to 690 of SEQ ID NO:5.

27. Process according to claim 25 for the preparation of a single-chain recombinant antibody designated Fv(FRP5)-ETA comprising a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO: 10.

28. Process according to claim 22 for the preparation of a single-chain recombinant antibody comprising the heavy chain variable domain of the mouse monoclonal antibody FWP51, the 15 amino acid polypeptide consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser, the light chain variable domain of the mouse monoclonal antibody FWP51, and an enzyme or a toxin, or a biologically active variant thereof.

29. Process according to claim 28 for the preparation of a single-chain recombinant antibody designated Fv(FWP51)-ETA comprising a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO: 11.

30. Process for the preparation of a mouse monoclonal antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 selected from the group consisting of antibodies designated FRP5, FSP16, FSP77 and FWP51 characterized in that cells producing such an antibody are multiplied in vitro or in vivo and, when required, the obtained antibody is isolated.

31. A process according to claim 30 for the preparation of a monoclonal antibody designated FRP5.

32. A process according to claim 30 for the preparation of a monoclonal antibody designated or FWP51.

33. A process for the preparation of a hybridoma cell line secreting monoclonal antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2 selected from the group consisting of antibodies designated FRP5, FSP16, FSP77 and FWP51 , characterized in that a suitable mammal is immunized with purified c-erbB-2 protein, an antigenic carrier containing purified c-erbB-2 or with cells bearing c-erbB-2, antibody-producing cells of the immunized mammal are fused with cells of a suitable myeloma cell line, the hybrid cells obtained in the fusion are cloned, and cell clones secreting the desired antibodies are selected.

34. A process for the preparation of a DNA comprising an insert coding for a recombinant antibody obtainable according to claim 1 comprising the steps of
    a) preparing murine DNA from the genome of a suitable hybridoma cell line and selecting the desired DNA coding for the variable heavy and/or light chain domains of the antibody with the desired specificity,
    b) preparing DNA coding for the desired signal sequence and/or preparing DNA coding for an effector molecule,
    c) synthesizing DNA coding for the desired spacer group by chemical methods,
    d) constructing recombinant genes encoding the recombiant antibodies by incorporating the DNA of step a) and, optionally, b) and/or c) into appropriate hybrid vectors,
    e) transferring the obtained hybrid vectors into a recipient host cell or retrieving the DNA coding for the recombinant genes and transferring the unlinked DNA into a recipient host cell,
    f) selecting and culturing the transformed host cell, and
    g) optionally isolating the desired DNA.

35. A process according to claim 34 for the preparation of a DNA comprising an insert coding for a heavy chain murine variable domain of a monoclonal antibody selected from the group consisting of antibodies FRP5, FSP16, FSP77 and FWP51 or coding for an amino acid sequence homologous to said heavy chain variable domain.

36. A process according to claim 34 for the preparation of a recombinant DNA comprising an insert coding for a light chain murine variable domain of a monoclonal antibody selected from the group consisting of antibodies FRP5, FSP16, FSP77 and FWP51 or coding for an amino acid sequence homologous to said light chain variable domain.

37. A process according to claim 34 for the preparation of a recombinant DNA which is a hybrid vector further comprising an origin of replication or an autonomously replicating sequence, one or more dominant marker sequences and, optionally, expression control sequences, signal sequences and additional restriction sites.

38. A process according to claim 34 for the preparation of hybrid vector comprising a Simian virus promoter and the mouse Ig H or L chain enhancer.

39. A process for the preparation of a host cell transformed with a recombinant DNA obtainable according to claim 34 wherein suitable recipient cells are transformed with a hybrid vector comprising a DNA insert coding for a heavy chain murine variable domain and/or for a light chain murine variable domain of an antibody directed to the extracellular domain of the growth factor receptor c-erbB-2, an origin of replication or an autonomously replicating sequence, one or more dominant marker sequences and, optionally, expression control sequences, signal sequences and additional restriction sites, and the transformed cells are selected.

40. A process for the preparation of a host cell according to claim 42 wherein the host cell is a cell strain of E. coli.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 92 81 0056

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X,D	WO-A-8 906 692 (GENENTECH, INC) 27 July 1989 * the whole document *	1,45,47, 49,51,52	C12N15/13 C12P21/08 C12N1/21 G01N33/574 A61K39/395
X	WO-A-9 014 357 (GENENTECH, INC.) 29 November 1990 * the whole document *	1,45,47, 49,51,52	
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 87, no. 21, 1 November 1990, WASHINGTON US pages 8291 - 8295; R.J.KREITMAN ET AL.: 'The recombinant immunotoxin anti-Tac(Fv)-Pseudomonas exotoxin 40 is' * the whole document *	15-29	
A,D	WO-A-8 911 533 (THE UNITED STATES OF AMERICA) 30 November 1989 * example 1 *	15-29	
A	SCIENCE. vol. 240, 20 May 1988, LANCASTER, PA US pages 1038 - 1041; A.SKERRA AND A. PLUCKTHUN: 'Assembly of a functional immunoglobulin Fv fragment in Escherichia coli' * figure 1 *	18	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

C07K
C12N
C12P
G01N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	07 MAY 1992	CUPIDO M.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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(54) Title: MONOCLONAL ANTIBODY AGAINST LPS CORE

(57) Abstract

By use of the Köhler/Milstein procedure involving immunization of mice with a number of different rough strains of heat-killed Gram-negative bacteria followed by fusion and suitable screening of the resulting hybridomas, murine monoclonal antibodies are obtained which are cross-protective against endotoxemia caused by at least two different Gram-negative bacterial strains having different core structures. The murine MAbs may be chimerized or humanized by known methods. The preferred product is a chimeric MAb of IgG isotype in which the hypervariable regions of the heavy chain have the amino acid sequences: Asp Tyr Tyr Met Thr; Leu Ile Arg Asn Lys Arg Asn Gly Asp Thr Ala Glu Tyr Ser Ala Ser Val Lys; and Gln Gly Arg Gly Tyr Thr Leu Asp Tyr; the hypervariable regions of the light chain have the amino acid sequences: Arg Ala Ser Gln Asn Ile Asn Ile Trp Leu Ser; Lys Ala Ser Asn Leu His Thr; and Leu Gln Gly Gln Ser Tyr Pro Arg Thr; the framework regions in the variable domains are murine and the constant domains are human.

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MONOCLONAL ANTIBODY AGAINST LPS CORE

This invention relates to the prevention, diagnosis and treatment of infectious diseases caused by Gram-negative bacteria and more particularly provides monoclonal antibodies (MAbs) against the lipopolysaccharide (LPS; also called endotoxin) constituent of the gram-negative bacterial membranes.

Enterobacteria are a widely prevalent group of Gram-negative microorganisms which cause serious and frequently lethal infections in patients undergoing certain types of surgery, anti-cancer chemotherapy or immunosupressive treatment or in patients suffering from various trauma, burns or wounds. The severity of the disease ranges from a preliminary, transient and limited episode of bacteremia to a subsequent, fulminant and life-threatening conditon of endotoxemia (also called septic shock) characterized, in particular, by a severe hypotension.

Some 425,000 cases of severe Gram-negative bacteremia occur yearly in the USA with an overall mortality of about 25%. The majority of these infections are due to the most common pathogen Escherichia coli, followed in frequency by Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus, Enterobacter and Serratia. All Gram-negative bacteria are characterized by a specific type of outer membrane which comprises a lipopolysaccharide (LPS) as major constituent. LPS plays an essential immunologic and physiopathologic role in the infections and is the major causative agent of septic shock.

Although the LPS constituent varies from one species to another, it may be generally described with reference to Figure 1 as consisting of three structural regions: Lipid A whose lipid portion is embedded in the outer leaflet of the outer membrane; the oligosaccharide core region and the O-specific outer region. Lipid A has the same basic structure in practically all enterobacteria and is the main endotoxic determinant. The core region shows a high degree of similarity among bacterial genera. It usually consists of a limited number of sugars. The inner core region is constituted of heptose and 2-Keto-3-deoxy-octonate (KDO) residues while the outer core region comprises galactose, glucose or N-acetyl-D-glucosamine residues displayed in various manners, depending upon the strain. For example, outer core structures R1 to R4 of different E. coli strains are shown in Figure 2. The O-specific outer region (also called O-specific side chain) is highly variable and is composed of repeated oligosaccharide units characteristic of the species. LPS molecules on the surface of a single cell do not have a constant amount of oligosaccharide units.

The presence of the O-specific side chain confers to a culture of a wild type bacterium a smooth aspect. This is the reason why wild type bacteria are usually referred to as smooth bacteria in contrast with rough mutants which lack the O-specific side chain and, sometimes, part of the core region and the cultures of which show a rough aspect. The different types of rough mutants from Salmonella are conventionally designated by the terms Ra, Rb, Rc, Rd and Re. As seen from Figure 1, the LPS of all of them comprises the lipid A structure while the Ra mutant is characterised by a complete core region, the Rb mutant is characterised by the absence of N-acetyl-D-glucosamine residue, the Rc mutant is characterised by the absence of N-acetyl-D-glucosamine and galactose residues, the Rd mutant is characterised by the absence of any residue constituting the outer core and the Re mutant is characterised by the sole KDO region attached to lipid A.

Since treatments for the toxic effect of LPS are not available,

attention has been focused on immunologic methods as an alternative or additional treatment to antibiotic therapy to prevent or control such infections. Current immunotherapy involves the administration of conventional polyclonal antisera and hyperimmune sera to bolster the native defenses of patients against the adverse effects of bacteria, for example, by enhancing opsonization and phagocytosis of the bacterial cells or by neutralization of the biological activity of LPS. However, the effectiveness of the antisera greatly varies depending upon a large number of factors including, for example, the composition and titre of the specific antibodies, which cannot be easily standardized.

To overcome the limited efficacy of serotherapy, it has been proposed to use cross-reactive MAbs. Cross-reactivity is of two kinds, which may be described as horizontal and vertical. By vertical cross-reactivity is meant that the MAb reacts with essentially all smooth LPS molecules of a particular bacterial strain, independent of the length of the O-specific side-chain. By horizontal cross-reactivity is meant that the MAb reacts with LPS having different core structures. This is necessary because therapy must be started as soon as the bacteremia has been empirically diagnosed, rather than waiting for the identification of the pathogen, which may take several days.

Such MAbs must recognize antigenic determinants located in the LPS structure which is shared by most enterobacteria i.e. Lipid A and the core region. They may be obtained by the well-known Kohler & Milstein method which, in particular comprises conventionally immunizing mice with an immunogen in which the inner antigenic epitopes of LPS are immediately available for raising antibodies. Suitable immunogens include heat-killed rough mutants of an enterobacterium e.g. the J5 strain of E. coli. Purified LPS is less suitable as an immunogen.

A MAb expected to be useful for preventing or treating bacteremia should not only be cross-reactive but also cross-protective against

the infections caused by the most common toxic bacteria. However, it has been reported in several scientific articles, for example, in Pollack et al, J. Infect. Dis. (1989) 159 (2): 168, that the large majority of antibodies raised against the conventional immunogens cited above cross-react poorly and, unfortunately fail to be protective against infections. MAbs have often been described as reactive on the basis of binding experiments involving rough rather than smooth LPS, and the lack of protectivity of these MAbs may be due to the fact that, in wild-type smooth LPS, the epitope for which the antibody is specific is not available, being hindered by the core region or the O-specific side chain. In particular, MAbs recognizing epitopes in the Lipid A part of the LPS molecule are generally ineffective.

It has now been found that monoclonal antibodies recognizing epitopes in the core region of the LPS molecule and having both vertical and horizontal cross-reactivity and also cross-protectivity can be obtained by modified and improved immunization and screening procedures. Such MAbs are initially obtained in murine form and may be converted by known recombinant DNA techniques into chimeric (murine variable region, human constant region) or humanized (murine hypervariable regions, human framework and constant region) forms.

Accordingly the present invention provides a monoclonal antibody which recognizes an epitope in the core region of the LPS molecule and which is cross-protective against endotoxemia caused by at least two different Gram-negative bacterial strains having different core structures.

Preferably the MAb recognises an epitope which is already present in the Rc core structure of E. coli and is also present in the complete core.

In E. coli, the MAb of the invention preferably reacts with all common smooth strain isolates, and preferably also with rough strain

mutants of all five core types (R1, R2, R3, R4, and K12). Preferably the MAb is also reactive with different strains of Salmonella.

In contrast to the immunization protocols described in the prior art, in which generally a single type of LPS (normally as heat-killed bacteria bearing the specific type of LPS) is used as immunogen, MAbs of the present invention may be produced by an immunization protocoll in which the animal to be immunized is exposed to a plurality of types of LPS molecule. This may be done either by immunization with a cocktail of different LPS types physically mixed together, or by immunizing in sequence by individual different LPS types. In both cases it is prefered to use heat-killed bacteria rather than purified LPS molecules. Other possible immunogens include bacteria killed by means other than heat (e.g. by formaldehyde) and LPS molecules linked to protein carriers.

The animal to be immunized is preferably a mouse, which may be of the Balb-c strain. It may however be preferable to use mice of different genetic background, for example New Zealand Black or Swiss Webster mice, which are capable of giving a wider immune response. The immunogen may be administered intravenously, or, preferably, subcutaneously, for example in the foot pad.

In a first preferred method, mice are immunized with a single cocktail of different strains of heat-killed bacteria, preferably rough strains having a complete core, for example a mixture of R1, R2, R3 and R4 strains of Ra E. coli. Alternatively two or more such cocktails, which may be different, may be given on different occasions. For example, injection of a mixture of E. coli R2 and R3 and Salmonella minnesota R60 may be followed a week later by a mixture of E. coli R1, R4 and 018 rough strain, and then the two injections repeated at further weekly intervals.

In a second preferred method, mice are immunized sequentially with a number of different rough strains of heat-killed bacteria, only

one strain being administered at any one time. For example mice may be immunized with Pseudomonas PAC 605 rough mutant followed by E. coli R1, R2 and R3 at monthly intervals.

Before any cell fusion is carried out between mouse myeloma cells and spleen cells from the immunized animal, there is preferably an initial screening step in which the strength and plurality of the immune response of the immunized animal is evaluated by testing the serum of the animal. Animals showing a strong immune response are subjected to a booster immunization and the spleen cells of these strongly-responding and re-immunized mice are used for cell fusion to make hybridomas by the conventional Köhler-Milstein technique. The booster immunization is preferably by a cocktail of different rough strain E. coli, even if the primary immunization was carried out by the second preferred method (sequential administration).

The resulting hybridomas are then screened for the cross-reactivity of the antibodies they produce, using the standard ELISA and Western blotting methods described below. In contrast to prior art methods, an initial screening is preferably carried out using a series of mixtures of different smooth and rough LPS types to select those MAbs reacting with a wide range of LPS molecules. In this way, widely cross-reactive MAbs can already be identified at the initial screening stage. For example, each hybridoma supernatant may be screened by testing for reactivity in the ELISA assay with seven different LPS cocktails and a control, according to the following scheme:

1) Smooth strains: Ec04 + 06 + 016 + 018K
2) Smooth strains: Ec012 + 015 + 086
3) Rough complete core: EcR1 + R4
4) Rough complete core: EcR2 + EcR3 + EcK12 + Sm R60
5) Rc core: Ec J5 + St878
6) Rc/Rd/Re core: Sm R5 + Sm R7 + Sm R4 + Ec F515 + Sm R595
7) Lipid A : derived from Ec K12 & Sm R595

8) Negative control: BSA

(Ec = E. coli, Sm = Salmonella minnesota, St = S. typhimurium, BSA = bovine serum albumin)

MAbs found to have good cross-reactivity are then screened further to select those which are not only cross-reactive, but also cross-protective.
This may be done using the following in vitro bioassay:

Inhibition of LPS-induced IL-6 secretion by murine peritoneal macrophages

Several monokines including Tumor Necrosis Factor (TNF), IL-1 and IL-6 (also called Interferon-β2) mediate many of the pathophysiological events associated with gram-negative sepsis and its accompanying endotoxemia. These monokines are secreted by macrophages, both in vitro and in vivo, in response to LPS. A protective anti-LPS antibody blocks the macrophage stimulation as shown in the following assay:

Murine peritoneal cells are obtained by peritoneal lavage with 0.34 M sucrose in distilled water. Peritoneal cells are seeded at 5.10^5 cells/ml in 0.2 ml serum free medium (IMDM-ATL, Schreier and Tees, Immunological Methods, Vol. II, Acad. Press (1981):263) and cultured for 4 hrs at 37° C (i) in the presence or absence of LPS e.g. LPS from E. coli R1 (0.05 ng/ml); E. coli R2 (0.05 ng/ml); E. coli R3 (0.05 ng/ml) and E. coli R4 (0.05 ng/ml); and (ii) in the presence or absence of a purified, endotoxin-free antibody the final concentration of which ranges from 0.05 ng to 50 µg/ml. The supernatants are recovered and the amount of IL-6 present in the supernatants is then measured using the IL-6 dependent hybridoma cell-line B13.29 (Aarden et al., Eur. J. Immunol. 1987, 17, 1911) as follows:

B13.29 cells are seeded at $2.5x10^4$ cells/ml in serum free medium

and cultured for 72 hrs in the absence of IL-6 and in the presence or absence of culture supernatant. Aliquots of the cultures (200 µl/well) are distributed in flat bottomed microtitre plates. IL-6 concentration in the supernatants is calculated in relation to a standard curve of IL-6.

For the purposes of this patent specification, a MAb is regarded as being protective against a given LPS if it gives in the above assay a reduction of IL-6 secretion of at least 50% when tested at a concentration of 5 µg/ml, the concentration of purified LPS being 0.05 ng/ml for rough LPS and correspondingly higher for the less active smooth types. A MAb is cross-protective if it is protective against at least two LPS having different core structures. Preferred cross-protective MAbs are cross-protective against LPS from different bacterial genera.

Prefered MAbs of the invention are of the IgG isotype.

By the use of the above immunization and screening methods, a number of novel mouse anti-LPS antibodies have been found which cross-react with several LPS of different genera and exhibit substantial cross-protective activity and that it is possible to construct other LPS binding molecules derived from these monoclonal antibodies and having the same characteristics since they share regions which determine the binding specificity i.e. the hypervariable regions. In particular, four preferred murine monoclonals according to the invention are hereinafter designated WN1 222-5 (isotype IgG2a), WN1 58-9 (IgG2b), H1 61-2 (IgG1), and SZ27 19.16.07 (IgG2a). Of these, the first two are particularly preferred.

Natural immunoglobulins or antibodies comprise a generally Y-shaped molecule built up of two identical heavy chains and two identical light chains, and having an antigen-binding site at the end of each upper arm. The remainder of the structure, in particular the stem of the Y, mediates effector functions associated with the

immunoglobulins. The general structure of an antibody of the IgG class is shown schematically in Figure 3A. Both heavy and light chains comprise a variable domain and a constant part. An antigen binding site consists of the variable domain of a heavy chain (V_H) associated with the variable domain of a light chain (V_L). The variable domains of the heavy and light chains have the same general structure which is illustrated in Figure 3B.

More particularly, the antigen binding characteristics of an antibody are essentially determined by 3 specific regions in the variable domain of the heavy and light chains which are called hypervariable regions or complementary determining regions (CDRs). As shown in Figure 3B, these 3 hypervariable regions alternate with 4 framework regions, (FRs) whose sequences are relatively conserved and which are not directly involved in binding. The CDRs form loops and are held in close proximity by the framework regions which largely adopt a β-sheet conformation. The CDRs of a heavy chain together with the CDRs of the associated light chain essentially constitute each of the two antigen binding sites of the antibody molecule.

The determination as to what constitutes a FR or a CDR region is usually made by comparing the amino acid sequence of a number of antibodies raised in the same species. The general rules for identifying the CDR and FR regions are given in Table I.

Furthermore, it has been recently found that the contribution made by a light chain variable domain to the energetics of binding is small compared to that made by the associated heavy chain variable domain and that isolated heavy chain variable domains have an antigen binding activity of their own. Such molecules, now commonly referred to as single domain antibodies, may be regarded as having an antigen binding site, even in the absence of an associated V_L domain.

In view of the foregoing, the invention provides a LPS binding molecule which comprises at least one antigen binding site comprising

at least one domain which comprises in sequence, the hypervariable regions hCDR1, hCDR2 and hCDR3 ; (domains h222-5 and h58-9)
said hCDR1 having the amino acid sequence Asp Tyr Tyr Met Thr;
said hCDR2 having the amino acid sequence Leu Ile Arg Asn W Arg Asn Gly Asp Thr Ala Glu Tyr Ser Ala Ser Val X;
wherein W is Lys or Tyr and X is Lys or Arg;
said hCDR3 having the amino acid sequence Gln Gly Arg Gly Tyr Thr Leu Asp Tyr;
and direct equivalents thereof.

Preferred is the hypervariable region hCDR2 in which W is Lys and X is Lys (h222-5) or in which W is Tyr and X is Arg (h58-9). More preferred is the hypervariable region hCDR2 in which W is Lys and X is Lys.

In a first aspect of the invention, the LPS binding molecule comprises an antigen binding site comprising a single domain.

In a second aspect of the invention, the LPS binding molecule comprises at least one antigen binding site comprising:

a) a first domain comprising in sequence the hypervariable regions hCDR1, hCDR2 and hCDR3, as defined above and,

b) a second domain comprising in sequence the hypervariable regions lCDR1, lCDR2 and lCDR3; (domains l222-5 and l58-9 [l222-5 or l58-9 stands for light 222-5 or light 58-9])
said lCDR1 having the amino acid sequence Arg Ala Y Z Asn Ile Asn Ile Trp Leu Ser;
wherein Y is Ser or Arg and Z is Gln or Leu;
said lCDR2 having the amino acid sequence Lys Ala Ser Asn Leu His Thr;
said lCDR3 having the amino acid sequence Leu Gln Gly Gln Ser Tyr Pro Arg Thr;
and direct equivalents thereof.

Preferred is the hypervariable region lCDR1 in which Y is Ser and Z is Gln (l222-5) or in which Y is Arg and Z is Leu (l58-9). More preferred is the hypervariable region lCDR1 in which Y is Ser and Z is Gln.

Unless otherwise indicated, any polypeptide chain is hereinafter described as having an amino acid sequence starting at the N-terminal extremity and ending at the C-terminal extremity.

When the antigen binding site comprises both the first and second domains, these may be located on the same polypeptide molecule or, preferably, each domain may be on a different chain, the first domain being part of an immunoglobulin heavy chain or fragment thereof and the second domain being part of an immunoglobulin light chain or fragment thereof.

By "LPS binding molecule" is meant any molecule capable of binding to LPS. The binding reaction may be shown by standard methods (qualitative assays) such as an ELISA using purified LPS or heat treated bacteria or a Western blotting using purified LPS; with reference to a negative control test in which an antigen of unrelated origin, e.g. bovine serum albumin (BSA), is used. A complete description of the assays cited above is given below.

1. Detection of binding to purified LPS in an ELISA

Microtitre plates (flat bottomed; microtest III flexible assay plates; Becton Dickinson, Falcon 3912) are coated with purified LPS at 2 µg/ml in coating buffer (diethylenebarbituric acid-Na salt 30 mM, Na acetate 30 mM, NaCl 116 mM; pH 4.5). 50 µl aliquots of the LPS solution are distributed into each well. Unrelated protein (BSA, 2% in PBS pH 7.2/0.02% sodium azide) is used to determine non-specific binding. Plates are incubated for 1 hr at 37°C and then overnight at 4°C in a humidified chamber. Plates are washed 4 times with a washing

solution i.e. phosphate buffered saline (PBS) pH 7.2, 0.05% vol/vol Tween 20. 0.02% sodium azide. Plates are blocked with 250 µl/well of 2% BSA in PBS/sodium azide 0.02% for 3 hrs at room temperature. Plates are washed again.

Antibody solutions are prepared in PBS/BSA 2%/sodium azide 0.02% at various dilutions, e.g. 1 µg/ml, 100 ng/ml, 10 ng/ml and 1 ng/ml. 50 µl aliquots of these solutions are distributed in the wells of the precoated plates. Incubation is carried out overnight at room temperature. After 4 washes, 50 µl per well of biotinylated affinity purified goat anti-mouse IgG or IgM of the correct subclass specificity, e.g. anti-mouse IgG2a for WN1 222-5 and anti-mouse IgG2b for WN1 58-9 or anti-human IgG1 or IgM for a variation of WN1 222-5 $_{(hu\gamma M)}$ or WN1 222-5$_{(hu\gamma G1)}$ (final dilution 1/10'000 in PBS 2% BSA; Southern Biotechnology Associates) is added. Incubation is carried out for 4 hrs at room temperature. After 4 washes, 50 µl per well of streptavidin alkaline phosphatase conjugate (Jackson Immuno Research Laboratories; final dilution 1/10'000 in PBS, 2% BSA) is added; Incubation is carried out for 1 hr at room temperature. After 4 washes, 100 µl per well of paranitrophenol phosphate (PNPP) diluted at 1 mg/ml in diethanolamine buffer (diethanolamine 1M, $MgCl_2.6H_2O$ 0.5 mM, pH 9.8) is added. After 1 hr, absorbance is read at 405 nm using a Titertek Multiskan ELISA reader (MCC/340, Flowlabs).

Advantageously, the purified LPS which is used is selected from smooth, complete core, Rb or Rc LPS. Examples of smooth LPS are LPS extracted from E. coli 0111B4 (Difco), E. coli 0127B8 (Difco), E. coli 0128B12 (Difco), Salmonella typhimurium BO ag 0:4, 5, 12 (SH 4809) (Bio-carb). Suitable complete core LPS, Rb LPS and Rc LPS are respectively obtained from S. minnesota (List) and S. typhimurium SL 684 (Sigma).

Tables IIA, IIB, IIC, and IID show in tabular form the binding of antibodies WN1 222-5, WN1 58-9, H1 61-2 and SZ27 19.16.07 respectively to purified LPS from different strains of Gram-negative bacteria.

2. Detection of binding to heat killed bacteria

Precoated plates are prepared as described in 1. above, using heat killed bacteria ($0.5x10^8$ cells/ml) rather than purified LPS. The binding reaction is tested and detected as described in 1. above.

Advantageously, the bacteria are smooth wild type bacteria or rough Ra, Rb or Rc mutants.

Tables IIIA, IIIB, IIIC, and IIID show in tabular form the binding of antibodies WN1 222-5, WN1 58-9, H1 61-2 and SZ27 19.16.07 respectively to heat killed bacteria of various Gram-negative strains.

The bacteria listed in Tables II and III are mostly common clinical isolates. The bacteria and/or the corresponding LPS are commercially available or are available on request from Dr. I. Poxton, Dept. of Bacteriology, University of Edinburgh, Scotland, or from Dr H. Brade, Forschungsinstitut Borstel, Borstel, W. Germany.

As will be seen from Tables II and III, the minimum core structure required for recognition by the antibodies of the invention is Rc.

3. Detection of binding to LPS using Western blotting

10 µl aliquots of a LPS solution at 1 mg/ml are mixed with an equal volume of 0.1 M Tris-HCl buffer, pH 6.8 containing 1% (wt/vol) sodium deoxycholate (DOC), 20% (wt/vol) glycerin and 0.001% bromophenol blue, and then sonicated. The samples so prepared are loaded onto an electrophoresis gel (4% stacking gel; 14% running gel). The electrophoresis system which is used is a modified Laemmli system (DOC-PAGE; Komuro et al Chem. Pharm. Bull. (1988) 36: 1218) using a Mini Protean II dual slab cell apparatus (Bio Rad Laboratories). The

samples are run at a current of 18 mA until the indicator dye enters the separating gel. The current is then increased to 25 mA.

Blotting of the gel is carried out using a 0.45 µm pore size nitrocellulose membrane (Bio Rad Laboratories) and a transfer electrophoresis cell (Mini transblot electrophoretic transfer cell apparatus, Bio Rad Laboratories) at 60 V for 20 min. The blot is soaked in Tris buffer saline (TBS: 20 nM Tris-HCl, 0.1 mM NaCl; pH 7.5) 1% BSA for 1 hr at room temperature. The immunoblot is developed for 2 hrs at room temperature using an antibody preparation at 0.1 µg/ml in TTBS (TBS, 0.05% Tween 20) 1% BSA.

The blot is washed twice in TTBS and further incubated for 45 min at room temperature with a biotinylated goat anti-mouse IgG2a or IgG2b antibody (Southern Biotechnology associates) at a final dilution of 1/10'000 in TTBS, 1% BSA. After washing twice, streptavidin alkaline phosphatase conjugate (Jackson Immuno Research Laboratories), used at a dilution of 1/10'000 in TTBS/BSA 1%, is added. Incubation is carried out for 45 min at room temperature. After 3 washes, the BCIP/NBT alkaline phosphatase colour development solution is added as indicated by the manufacturer (Bio Rad Laboratories). In parallel, the gel is fixed by overnight incubation in a solution containing 40% ethanol and 5% acetic acid and is silver-stained according to the method of Tsai and Frash, Ann. Biochem. (1982) 119: 115.

In this assay, the antibodies of the invention show a binding reaction with LPS extracted either from smooth bacteria or from rough mutants. Particular experiments involving WN1 222-5, WN1 58-9, H1 61-2 and SZ27 19.16.07 are to be seen in Figures 4A; 4B, 4C and 4D respectively. The LPS content extracted from a smooth bacterium is separated by electrophoresis into bands corresponding to LPS molecules having different molecular weights, depending on the size of the O-specific side chain. These LPS molecules range from LPS molecules without any O-specific side chain to LPS molecules having 40 or more units in the side chain. The antibodies of the invention react with

rough repeating units and all these LPS molecules, containing O-side chain repeating units. This indicates that the epitope for which the LPS-binding molecules of the invention are specific is not hindered by the O-specific side chain. Therefore the majority of LPS molecules of a smooth bacterium are able to react with an LPS-binding molecule of the invention.

Examples of antigen binding molecules include immunoglobulin (Ig) molecules, e.g. antibodies as produced by B-cells or hybridomas and chimeric or humanized antibodies or fragments thereof, e.g. $F(ab')_2$ and Fab fragments, as well as single chain or single domain antibodies. Immunoglobulin molecules may be of different isotypes, for example IgG, IgM, IgA or IgE antibodies, of which IgG are preferred.

A single chain antibody consists of the variable domains of the antibody heavy and light chains of an Ig molecule covalently bound by a peptide linker usually consisting of from 10 to 30 amino acids, preferably from 15 to 25 amino acids. Therefore, such a structure does not include the constant part of the heavy and light chains and it is believed that the small peptide spacer is less antigenic than a whole constant part. By "chimeric antibody" is meant an antibody in which the constant regions of the heavy or light chain or both are of human origin while the variable domains of both heavy and light chains are of non-human (e.g. murine) origin. By "humanized antibody" is meant an antibody in which the hypervariable regions are of non-human (e.g. murine) origin, while all other parts of the immunoglobulin molecule, i.e. the constant regions and the highly conserved framework regions of the variable domains, are of human origin.

Hypervariable regions may be associated with any kind of framework regions, preferably of murine or human origin. Suitable framework regions are described in "Sequences of proteins of immunological interest", Kabat E.A. et al, US department of health and human services, Public health service, National institute of health. However, the preferred framework regions are those of WN1 222-5 or WN1

58-9, wherein the regions of WN1 222-5 are the most preferred.

Sequence Identifier No. 1 shows the complete amino acid sequence of the heavy chain variable domain of WN1 222-5, which consists, in sequence from the N-terminal, of framework regions hFR1, hFR2, hFR3 and hFR4 interspersed with the hypervariable regions hCDR1, hCDR2 and hCDR3, whose amino acid sequence is also stated above. In hCDR2 of WN1 222-5 W stands for Lys and X stands for Lys. Sequence Identifier No. 2 shows the complete amino acid sequence of the heavy chain variable domain of WN1 58-9, which consists, in sequence from the N-terminal, of framework regions $hFR1_r$; $hFR2_r$; $hFR3_r$ and hFR4 interspersed with the hypervariable regions hCDR1; hCDR2 and hCDR3, whose amino acid sequence is also stated above. In hCDR2 of WN1 58-9 W stands for Tyr and X stands for Arg. The index r after the FR stands for an amino acid sequence nearly identical to the amino acid sequence without index. The sequence with index comprises at least one replaced amino acid in contrast to the sequence without index.

Sequence Identifier No. 3 shows the complete amino acid sequence of the light chain variable domain of WN1 222-5, consisting in sequence of framework regions lFR1; lFR2; lFR3 and lFR4 interspersed with the hyper- variable regions lCDR1; lCDR2 and lCDR3 whose amino acid sequence is also stated above. In lCDR1 of WN1 222-5 Y stands for Ser and Z stands for Gln. Sequence Identifier No. 4 shows the complete amino acid sequence of the light chain variable domain of WN1 58-9, consisting in sequence of framework regions $lFR1_r$; $lFR2_r$; $lFR3_r$ and lFR4 interspersed with the hypervariable regions lCDR1, lCDR2 and lCDR3 whose amino acid sequence is also stated above. In lCDR1 of WN1 58-9 Y stands for Arg and Z stands for Leu.

The preferred heavy chain framework is hFR1; hFR2; hFR3 and hFR4 as shown in Seq. Id. No. 1 and the preferred light chain framework is lFR1; lFR2; lFR3 and lFR4 as shown in Seq. Id. No. 3.

Accordingly, the invention also provides an LPS binding molecule which comprises at least one antigen binding site comprising either a

domain having an amino acid sequence substantially identical to that shown in Seq. Id. No. 1 or alternatively No. 2 starting with amino acid at position 1 and ending with amino acid at position 120; or a first domain as described above and a second domain having an amino acid sequence substantially identical to that shown in Seq. Id. No. 3 or alternatively 4, starting with amino acid at position 1 and ending with amino acid at position 107.

Monoclonal antibodies raised against a toxic antigen must necessarily be developed in a non-human system e.g. in mice. As a direct consequence of this, a xenogenic antibody as produced by a hybridoma, when administered to humans, elicits an undesirable immune response which is essentially mediated by the constant part of the xenogenic immunoglobulin. This clearly limits the use of such antibodies as they cannot be administered over a prolonged period of time. Therefore it is particularly preferred to use single chain antibodies or chimeric or humanized monoclonal antibodies which are less likely to elicit a substantial allogenic response when administered to humans.

In view of the foregoing, a more preferred LPS binding molecule of the invention is selected from a chimeric anti-LPS antibody which comprises at least

a) one immunoglobulin heavy chain or fragment thereof which comprises

(i) a variable domain comprising in sequence the hypervariable regions hCDR1, hCDR2 and hCDR3 as shown in Seq. Id. No. 1 or No. 2 and

(ii) the constant part or fragment thereof of a human heavy chain; and,

b) one immunoglobulin light chain or fragment thereof which comprises

(i) a variable domain comprising in sequence the hypervariable regions lCDR1; lCDR2 and lCDR3 as shown

in Seq. Id. No. 3 or No. 4 and

(ii) the constant part or fragment thereof of a human light chain;

and direct equivalents thereof.

Alternatively, a LPS binding molecule of the invention may be selected from a single chain binding molecule which comprises an antigen binding site comprising

a) a first domain comprising in sequence the hypervariable regions hCDR1, hCDR2 and hCDR3, as shown in Seq. Id. No. 1 or No. 2,

b) A second domain comprising in sequence the hypervariable regions lCDR1, lCDR2 and lCDR3, as shown in Seq. Id. No. 3 or No. 4 and

c) a peptide linker which is bound either to the N-terminal extremity of the first domain and to the C-terminal extremity of the second domain or to the C-terminal extremity of the first domain and to the N-terminal extremity of second domain;

and direct equivalents thereof.

As is well known, minor changes in an amino acid sequence such as deletion, addition or substitution of one or several amino acids may lead to an allelic form of the original protein which has substantially identical properties. Thus, by the term "direct equivalents thereof" is meant either any single domain LPS binding molecule (molecule X)

(i) in which the hypervariable regions taken as a whole are at least 80% homologous, preferably at least 90% homologous, more preferably at least 95% homologous to the hypervariable regions hCDR1, hCDR2 and hCDR3 as shown in Seq. Id. No. 1 or 2 and,

(ii) which is capable of binding to LPS substantially to the same extent as a reference molecule having framework regions

identical to those of molecule X but having hypervariable regions hCDR1, hCDR2 and hCDR3 identical to those shown in Seq. Id. No. 1 or No. 2;

or any LPS binding molecule having at least two domains per binding site (molecule X')

(i) in which the hypervariable regions taken as a whole are at least 80% homologous, preferably at least 90% homologous, more preferably at least 95% homologous to the hypervariable regions hCDR1, hCDR2, hCDR3, lCDR1, lCDR2 and lCDR3 as shown in Seq. Id. No. 1; 2; 3 and 4, and

(ii) which is capable of binding to LPS substantially to the same extent as a reference molecule having framework regions and constant parts identical to molecule X' but having hypervariable regions hCDR1, hCDR2, hCDR3, lCDR1, lCDR2 and lCDR3 identical to those shown in Seq. Id. No. 1; 2; 3 and 4.

One LPS binding molecule may be considered as binding to LPS substantially to the same extent as another if the two molecules can be shown effectively to compete with each other in competitive ELISA binding assays on different LPS molecules, for example on the LPS from E. coli J5 and from Salmonella Ra 60 and if the binding affinities of the two molecules vary from each other in each case by a factor of not more than 100, preferably not more than 10.

Most preferably, the chimeric anti-LPS antibody comprises at least

a) one heavy chain which comprises a variable domain having an amino acid sequence substantially identical to that shown in Seq. Id. No. 1 starting with amino acid at position 1 and ending with amino acid at position 120 and the constant part of a human heavy chain; and

b) one light chain which comprises a variable domain having an amino acid sequence substantially identical to that shown in Seq. Id. No. 3 starting with amino acid at position 1 and ending with amino acid at position 107 and the constant part of a human light chain.

The constant part of a human heavy chain may be of the γ_1, γ_2, γ_3, γ_4, μ, α_1, α_2, δ or ε type, preferably of the γ type, more preferably of the γ_1 type, whereas the constant part of a human light chain may be of the κ or λ type, preferably of the κ type. The amino acid sequence of all these constant parts are given in Kabat et al. (supra).

Conjugates of the LPS binding molecules of the invention, e.g. enzyme or toxin conjugates, are also included within the scope of the invention, as are LPS binding molecules labelled with radioactive isotopes or fluorescent markers.

A LPS binding molecule of the invention may be produced by recombinant DNA techniques. In view of this, one or more DNA molecules encoding the binding molecule must be constructed, placed under appropriate control sequences and transferred into a suitable host organism for expression.

In a very general manner, there are accordingly provided

(i) DNA molecules encoding a single domain LPS binding molecule of the invention, a single chain LPS binding molecule of the invention, a heavy or light chain or fragment thereof of a LPS binding molecule of the invention and

(ii) the use of the DNA molecules of the invention for the production of a LPS binding molecule of the invention by recombinant means.

The present state of the art is such that the skilled man will be

able to synthetize the DNA molecules of the invention given the information provided herein i.e. the amino acid sequences of the hypervariable regions and the DNA sequences coding for them. A method for constructing a variable domain gene is for example described in EPA 239 400 and may be briefly summarized as follows: A gene encoding a variable domain of a MAb of whatever specificity is cloned. The DNA segments encoding the framework and hypervariable regions are determined and the DNA segments encoding the hypervariable regions are removed so that the DNA segments encoding the framework regions are fused together with suitable restriction sites at the junctions. Double stranded synthetic CDR cassettes are prepared by DNA synthesis according to the sequences given in Seq. Id. No. 1; 2; 3 or 4. These cassettes are provided with sticky ends so that they can be ligated at the junctions of the framework. A protocol for achieving a DNA molecule encoding an immunoglobulin variable domain is shown in Figure 5.

Furthermore, it is not necessary to have access to the mRNA from a producing hybridoma cell line in order to obtain a DNA construct coding for the MAbs of the invention. Thus PCT application WO 90/07861 gives full instructions for the production of a MAb by recombinant DNA techniques given only written information as to the nucleotide sequence of the gene. The method comprises the synthesis of a number of oligonucleotides, their amplification by the PCR method, and their splicing to give the desired DNA sequence.

Expression vectors comprising a suitable promoter and genes encoding heavy and light chain constant parts are publicly available. Thus, once a DNA molecule of the invention is prepared it may be conveniently trans- ferred in an appropriate expression vector. DNA molecules encoding single chain antibodies may also be prepared by standard methods, for example, as described in WO 88/1649.

In view of the foregoing and since the mouse MAb as naturally secreted by the hybridoma is not the preferred type of MAb, it is

considered that, although no deposit has been made of the hybridoma producing WN1 222-5 or WN1 58-9, nevertheless the present application discloses the invention in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art.

In a particular embodiment of the invention, the recombinant means for the production of a LPS binding molecule includes first and second DNA constructs as described below:

The first DNA construct encodes a heavy chain or fragment thereof and comprises

a) a first part which encodes a variable domain comprising alternately framework and hypervariable regions, said hypervariable regions being in sequence hCDR1, hCDR2 and hCDR3, the amino acid sequences of which are shown in Seq. Id. No. 1 or 2; this first part starting with a codon encoding the first amino acid of the variable domain and ending with a codon encoding the last amino acid of the variable domain, and

b) a second part encoding a heavy chain constant part or fragment thereof which starts with a codon encoding the first amino acid of the constant part of the heavy chain and ends with a codon encoding the last amino acid of the constant part or fragment thereof, followed by a non-sense codon.

Preferably, this first part encodes a variable domain having an amino acid sequence substantially identical to the amino acid sequence as shown in Seq. Id. No. 1 or 2 starting with the amino acid at position 1 and ending with the amino acid at position 120. More preferably the first part has the nucleotide sequence as shown in Seq. Id. No. 1 or 2 starting with the nucleotide at position 1 and ending with the nucleotide at position 361. Also preferably, the second part encodes the constant part of a human heavy chain, more preferably the constant part of the human γ1 chain. This second part may be a DNA

fragment of genomic origin (comprising introns) or a cDNA fragment (without introns). The sequence of Sequence Identifier 1 is more preferred than the sequence of Sequence Identifier No. 2

The second DNA construct encodes a light chain or fragment thereof and comprises

a) a first part which encodes a variable domain comprising alternately framework and hypervariable regions; said hypervariable regions being in sequence 1CDR1, 1CDR2 and 1CDR3, the amino acid sequences of which are shown in Seq. Id. No. 3 or 4; this first part starting with a codon encoding the first amino acid of the variable domain and ending with a codon encoding the last amino acid of the variable domain, and

b) a second part encoding a light chain constant part or fragment thereof which starts with a codon encoding the first amino acid of the constant part of the light chain and ends with a codon encoding the last amino acid of the constant part or fragment thereof followed by a non-sense codon.

Preferably, this first part encodes a variable domain having an amino acid sequence substantially identical to the amino acid sequence as shown in Seq. Id. No. 3 or 4 starting with the amino acid at position 1 and ending with the amino acid at position 107. More preferably, the first part has the nucleotide sequence as shown in Seq. Id. No. 3 or 4 starting with the nucleotide at position 1 and ending with the nucleotide at position 336. Also preferably the second part encodes the constant part of a human light chain, more preferably the constant part of the human κ chain.

In the first and second DNA constructs, the first and second parts are preferably separated by an intron. In the intron located between the first and second part, an enhancer is preferably inserted. The presence of this genetic element, which is transcribed but not

translated, may be required for an efficient transcription of the second part. More preferably the first and second DNA constructs comprise the enhancer of a heavy chain gene.

The first or second DNA construct advantageously comprises a third part which is located upstream of the first part and which encodes a leader peptide. This peptide is required for secretion of the chains by the host organism in which they are expressed and is subsequently removed by the host organism. Preferably, the third part of the first DNA construct encodes a leader peptide of a heavy chain. Also preferably, the third part of the second DNA construct encodes a leader peptide of a light chain. Suitable leader peptides are indicated in Kabat et al. (supra). The structure of genes encoding the heavy and light chain of an Ig molecule is shown diagramatically in Figure 3A.

Each of the DNA constructs are placed under the control of suitable control sequences, in particular under the control of a suitable promoter. Any kind of promoter may be used, provided that it is adapted to the host organism in which the DNA constructs will be transferred for expression. However, if expression is to take place in a mammalian cell, it is particularly preferred to use the promoter of an immunoglobulin gene.

The desired antibody may be produced in a cell culture or in a transgenic animal. A suitable transgenic animal may be obtained according to standard methods which include microinjecting the first and second DNA constructs, placed under suitable control sequences, into fertilized ova, transferring the so prepared ova into appropriate pseudo-pregnant females and selecting a descendant expressing the desired antibody.

When the antibody chains are to be produced in a cell culture, the DNA constructs are advantageously inserted together or separately in an expression vector, the latter possibility being preferred. More

preferably, they are separately inserted on two different but mutually compatible expression vectors.

Accordingly, the invention also provides an expression vector able to replicate in a prokaryotic or eukaryotic cell line which comprises at least one of the DNA constructs above described.

The next stage is the transfer of the expression vector or vectors containing the DNA constructs into a suitable host organism. When the DNA constructs are separately inserted on two expression vectors, they may be transferred separately, i.e. one type of vector per cell, or co- transferred, this latter possibility being preferred. A suitable host organism may be a bacteria, a yeast or a mammalian cell line, the last of these being preferred. More preferably, the mammalian cell line is of lymphoid origin e.g. a myeloma, hybridoma or a normal immortalized B-cell, but does not express any endogeneous antibody heavy or light chain.

It is also preferred that the host organism contains a large number of copies of the vectors per cell. If the host organism is a mammalian cell line, this desirable goal may be reached by amplifying the number of copies according to standard methods. Amplification methods usually consist of selecting for increased resistance to an antibiotic, said resistance being encoded by the expression vector.

In another aspect of the invention, there is provided a process for producing a multi-chain LPS binding molecule which comprises (i) culturing an organism which has been transformed with the first and second DNA constructs of the invention and (ii) recovering an active LPS binding molecule from the culture.

Alternatively, the heavy and light chains may be separately recovered and reconstituted into an active binding molecule after in vitro refolding. Reconstitution methods are well-known in the art; Examples of methods are in particular provided in EPA 120 674 or in

EPA 125 023.

Therefore a process may also comprise

(i) culturing a first organism which is transformed with a first DNA construct of the invention and recovering said heavy chain or fragment thereof from the culture and

(ii) culturing a second organism which is transformed with a second DNA construct of the invention and recovering said light chain or fragment thereof from the culture and

(iii) reconstituting in vitro an active LPS binding molecule from the heavy chain or fragment thereof obtained in (i) and the light chain or fragment thereof obtained in (ii).

In a similar manner, there is also provided a process for producing a single chain or single domain LPS binding molecule which comprises (i) culturing an organism which is transformed with a DNA construct respectively encoding a single chain or single domain LPS binding molecule of the invention and (ii) recovering said molecule from the culture.

In the processes of the invention, it is most preferred that the DNA constructs are inserted into expression vectors.

LPS binding molecules of the invention exhibit very good protective activity against LPS of Gram-negative endotoxemia as shown both in the in vitro IL-6 assay described above, and in the following in vivo bioassay.

Rabbit pyrogen model

Rabbits are weighed and placed in restraining boxes. Probes from the APT 75 (Automatic Pyrogen Test Processor) are inserted in the rectum of each rabbit. The temperature of each rabbit is monitored

every 15 minutes from 5 minutes after probe insertion, for an initial period of 95 minutes to establish a base/initial temperature (the base is the mean of the last three readings; if these show a greater than 0.3° range of fluctuation the test is not initialised).

Rabbits are then injected in a marginal ear vein with the LPS-binding molecule followed 30 min to 2 hr later by LPS in the same ear vein. LPS from different E. coli and salmonella, e.g. Salmonella abortus equi may be used. The suitable dose of LPS-binding molecule is to be determined, depending upon the type of molecule. For example WN1 222-5 is administered at 1 mg to 5 mg per kg body weight. For injection, this antibody is also prepared at 1 mg/ml in pyrogen-free saline and the LPS is injected at 10-100 ng/kg body weight, depending on the LPS used.

Control animals receive either LPS alone or the antibody alone. Rabbits are monitored at 15 min. intervals for a period starting from the injection and not exceeding 300 min.

The percentage of inhibition is measured as follows:

$$\% \text{ inhibition} = 100 - \frac{(\Delta T \text{ for Ab and LPS}) - (\Delta T \text{ for Ab alone})}{(\Delta T \text{ for LPS alone})} \times 100$$

ΔT = Temperature rise

In this assay, LPS binding molecules of the invention significantly reduce the increase of temperature in comparison with the negative control (LPS alone). Depending upon the type of LPS, the % of inhibition may reach levels well above 50%. A protective MAb may be defined in terms of this in vivo assay as one which gives at least 30% inhibition of fever 240 min after an LPS challenge of 10-100 ng/kg with an antibody dose of 1-5 mg/kg.

Therefore the invention also provides

(i) the use of an LPS binding molecule of the invention for preventing or treating gram-negative endotoxemia in humans

(ii) a method of preventing or treating gram-negative endotoxemia in humans which comprises administering an effective amount of an LPS binding molecule of the invention to a patient in need of such treatment.

(iii) a pharmaceutical composition for preventing or treating Gram-negative bacterial infections in humans which comprises an LPS binding molecule of the invention and a pharmaceutically acceptable carrier or diluent.

For these indications, the appropriate dosage will, of course, vary depending upon, for example, the particular molecule of the invention to be employed, the host, the mode of administration and the nature and severity of the condition being treated. However, in therapeutic use, satisfactory results are generally indicated to be obtained by administering at repeated intervals e.g. every two days or twice a week doses of from about 0.1 mg to about 15 mg per kilogram body weight as long as the patient is at risk. A molecule of the invention is conveniently administered parenterally, normally intravenously, for example, into the anticubital or other peripheral vein. A prophylactic treatment typically comprises administering a single dose of a molecule of the invention at a dosage of from about 20 µg to about 5 mg per Kg body weight.

Pharmaceutical compositions of the invention may be manufactured in conventional manner. A composition according to the invention is preferably provided in lyophilized form. For immediate administration it is dissolved in a suitable aqueous carrier, for example sterile water for injection or sterile buffered physiological saline. If it is considered desirable to make up a solution of larger volume for administration by infusion rather as a bolus injection, it is advantageous to incorporate human serum albumin or the patient's own

heparinised blood or other sugar stabilisers into the saline at the time of formulation. The presence of an excess of such physiologically inert protein prevents loss of monoclonal antibody by adsorption onto the walls of the container and tubing used with the infusion solution. If albumin is used, a suitable concentration is from 0.5 to 4.5% by weight of the saline solution.

LPS binding molecules of the invention, either unlabelled or, preferably, labelled with a radioactive isotope or a fluorescent marker, may also be used for diagnostic purposes to determine the nature, location and extent of Gram-negative bacterial infections, or analytically to detect the presence of LPS or Gram-negative bacterial contamination in water, foodstuffs, biological fluids, etc. Thus for example a labelled LPS binding molecule of the invention may be useful for the imaging of localised infectious foci for surgical removal or other treatment. The LPS binding molecules of the invention may also be attached to a solid phase support-material to form the solid phase of an affinity chromatography purification system for the removal of LPS molecules from biological fluids, e.g. blood serum.

Brief description of the drawings

Figure 1 shows the detailed structure of a Salmonella LPS molecule, indicating the various Ra-Re rough mutant types. In this Figure, Abe = abequose, Ac = acetyl, Ara = 4-amino-4-deoxy-L-arabinose, Etn = ethanolamine, FA = hydroxy fatty acid, Gal = D-galactose, Glc = D-glucose, GlcN = D-glucosamine, GlcNAc = N-acetyl-d-glucosamine, Hep = heptose, KDO = 2-keto-3-deoxyoctonic acid, Man = mannose, P = phosphate, Rha = L-rhamnose. Dotted lines indicate incomplete substitution.

Figure 2 is a representation of the outer core structures R1; R2; R3; R4 and K12 of different E. coli strains. The same abbreviations are used as in Fig. 1.

Figure 3A is a schematic diagram showing the structure of an IgG molecule as well as the genes encoding heavy and light chains respectively designated (1) and (2). Figure 3B schematically represents the arrangement of a variable domain of a heavy or light chain into framework (FR) and hypervariable (CDR) regions.

Figure 4A shows the binding capacity of monoclonal antibody WN1 222-5 against different LPS molecules derived from eight different E. coli strains as determined by Western blotting. The drawing represents the spots of the gel. The strains are described in detail in Tables II & III and the lane numbering represents: St = standard; 1 = E. coli 0111B4; 2 = E. coli 086; 3 = E. coli 018K$^-$; 4 = E. coli 016; 5 = E. coli 015; 6 = E. coli 012; 7 = E. coli 06; and 8 = E. coli 04.

Figure 4B shows the binding capacity of WN1 58-9 against different LPS molecules derived from eight different bacterial strains as determined by Western blotting. The drawing represents the spots of the gel. The lane numbering represents: St = standard; 1 = S. minnesota wild type; 2 = E. coli 018; 3 = E. coli 016; 4 = E. coli 015; 5 = E. coli 012; 6 = E. coli 06; 7 = E. coli 04; and 8 = E. coli 02.

Figure 4C shows the binding capacity of H1 61-2 against different LPS molecules derived from eight different bacterial strains as determined by Western blotting. The drawing represents the spots of the gel. The lane numbering represents: St = standard; 1 = S. minnesota wild type; 2 = E. coli 018K$^-$; 3 = E. coli 04; 4 = E. coli 06; 5 = E. coli 012; 6 = E. coli 015; 7 = E. coli 016; and 8 = E. coli 086.

Figure 4D shows the binding capacity of SZ27 19.16.07 against different LPS molecules derived from eight different bacterial strains as determined by Western blotting. The drawing represents the spots of the gel. The lane numbering represents: St = standard; 1 = E. coli

04; 2 = E. coli 016; 3 = E. coli 018K⁻; 4 = K235; 5 = R_1B; 6 = R_2B; 7 = R_3B and 8 = R_4B.

Figure 5 shows a protocol for constructing CDR replacements by insertion of CDR cassettes into a vector containing 4 framework regions fused together.

Figures 6A and 6B show the parental expression vectors pSV-2 neo and pSV-2DHFR. Both plasmids comprise an ampicillin resistance gene (amp^R) and the origin of replication of pBR322 and SV40 (pBR322 ori and SV40 or). pSV-2neo is characterized by the presence of a neomycin gene (neo^R) and the gene encoding the human γ_1 constant part (hu $C\gamma_1$) while pSV-2 DHFR has inserted a dihydrofolate reductase (DHFR) gene (methotrexate resistance) and the gene encoding the human κ constant part (hu C_κ). The final vectors for expressing the chimeric heavy or light chain are respectively obtained by inserting into pSV-2neo a DNA fragment encoding the leader peptide (L), and the variable domain (VDJ_4) of the WN1 222-5 heavy chain together with the mouse heavy chain enhancer and by inserting into pSV2-DHFR a DNA fragment encoding the leader peptide (L) and the variable domain (VJ_1) of the WN1 222-5 light chain together with the mouse heavy chain enhancer.

Figure 7 shows a drawing of the cloning vector p Bluescript II SK^- and p Bluescript SK^+ (Stratagene).

The following Examples illustrate the invention:

Example 1: Preparation of Murine Monoclonal Antibody WN1 222-5

a) Immunization Procedure

New Zealand Black mice were immunized i.v. with 10^8 heat-killed baceria in 0.1 ml. Four injections were carried out, as follows:

week 1 EcR2 + EcR3 + SmR60
week 2 EcR1 + EcR4 + Ec018 rough strain
week 3 EcR2 + EcR3 + SmR60
week 4 EcR1 + EcR4 + Ec018 rough strain

Antibody responses were monitored in tail bleed samples, and a mouse was selected for boosting on the basis of its strong plural response profile.

After one month, two injections, one day apart, of a cocktail of the 6 different strains (10^8 heat-killed bacteria) were given, the first injection i.v., the second i.p.

b) Fusion

On the fourth day after boosting, spleen cells were recovered and fused with the non-secreting murine B cell lymphoma PAI-0 cell line, using standard procedures. Supernatant from wells containing growing hybridomas were screened using cocktails of different smooth and rough LPS as described above, and hybridomas producing cross-reactive MAb's were cloned.

One of the resulting clones was WN1 222-5, which secretes a murine MAb of the IgG2ak isotype. The WN1 222-5 MAb was purified from culture supernatants collected after in-vitro fermentation of the WN1 222-5 clone and made pyrogen-free by treatment with

detergent.

The reactivity pattern of WN1 222-5 is shown in Tables II A and III A.

Example 2: Preparation of Murine Monoclonal Antibody WN1 58-9

WN1 58-9 is a further clone obtained from the fusion described in Example 1. Its reactivity pattern is shown in Tables II B and III B.

Example 3: Preparation of Murine Monoclonal Antibody H1 61-2

Balb/c mice were immunized i.v. with a cocktail of EcK12, EcR2 and EcR3 (10^8 heat-killed bacteria, four injections one week apart). Antibody responses were monitored in tail bleed samples, and a mouse was selected for boosting on the basis of its strong plural response profile.

After one month, two injections, one day apart, of a cocktail of the three different strains (10^8 heat-killed bacteria) were given. The first injection was i.v., the second i.p. On the fourth day the spleen cells were fused with the PAI-0 cell line using standard procedures.

Primary screening was carried out using the following cocktails of different rough LPS:

1) complete core: EcR2, EcR3, EcK12
2) complete core: EcR1, EcR4, SmR60
3) Rb2 : SmR345
4) Rc : EcJ4, St878, SmR5
5) Rd : SmR7, SmR4
6) Re : EcK12, StSL1102, StSL1181, SmR595
7) Lipid A : EcK12, SmR595
8) Negative Control: BSA

Hybridomas producing cross-reactive MAb's were cloned, and one of

the resulting clones was H1 61-2, which secretes a murine MAb of the IgG1k isotype. The H1 61-2 MAb was purified from culture supernatants, and gave the reactivity pattern shown in Tables IIC and III C.

Example 4: Preparation of Murine Monoclonal Antibody SZ 27 19.16.07

Balb/c female mice were immunized with 10^8 heat-killed bacteria in 0.1 ml i.v. on each of six days (day 0, 1, 2, 7, 8 and 9). Different immunogens were used, at 28 day intervals between starting each immunogen. The immunogens used were

1st	6 injections	P. aeruginosa PAC-605
2nd	" "	EcR1
3rd	" "	EcR2
4th	" "	EcR3

A group of 5 mice received identical immunizations. Antibody responses were monitored in tail bleed samples to purified LPS antigen from the following strains:

S. typh. Ra*, Rb*, Rc*, Rd and Re
S. Minnesota Lipid A
E. coli R1*, C61*, K12, Re (strain D31m4)
and Lipid A (ex D31m4)
P. aeruginosa C605*

Strong antibody responses to the marked antigens * had developed after cyclic immunization with 4 different bacteria, and a mouse was selected for boosting on the basis of its strong plural response profile.

Six weeks after completion of the last series of immunization, the selected mouse was boosted i.v. with a cocktail of 2.10^8 heat-killed bacteria of each of E. coli R1, R2, R3, R4 and K12. The spleen was removed three days later for fusion.

Fusion was carried out with the NS-0 cell line using standard procedures.

Primary screening was carried out using two LPS cocktails:

1) S. minnesota Ra + Rc + Re
2) E. coli C62 + K12 + Re

260 hybridoma supernatants were screened, and 20 of those showing strong responses to both cocktails were selected for further growth. These were then given a secondary screening on 11 different LPS antigens before selection for cloning. These were:

S. typh. Ra, Rb, Rc, Rd and Re
S. minn. Lipid A
E. coli R1, K12, Re, Lipid A
P. aerug. C605.

A number of hybridomas, including SZ27 19 showed the following reaction patterns:

strong - S. typh Ra; E. coli R1
weak - S. typh Rb, Rc; P. aerug. 602
negative - S. typh Rd, Re; E. coli lipid A, K12, Re

After subcloning, the clone SZ27 19.16.07 was isolated. It produced a murine MAb of the IgG2ak isotype.

The reaction pattern of this antibody is shown in Tables II D and III D.

Example 5: Cloning of the WN1 222-5 or WN1 58-9 heavy chain variable region by Polymerase Chain Reaction (PCR) and construction of a chimeric gene

Cloning step No. 1

The amino terminal sequence of the heavy chain is determined as being Glu-Val-Lys-Leu-Val-Glu-Ser-Gly. Based on this an upstream primer complementary to the mRNA encoding the end of the expected leader sequence and the amino acid sequence cited above is constructed so that its nucleotide sequence reads:

Sal 1

5′ AGGT GTC GAC TCC GAG GTG AAG CTG GTG GAG TCT GG 3′

Glu Val Lys Leu Val Glu Ser Gly

A downstream primer complementary to the mRNA encoding a fragment of the mouse γ2a constant part is also constructed so that its nucleotide sequence reads 5′ TCCAGGTCAAGGTCACTG 3′.

The upstream and downstream primers are used together to amplify a DNA fragment encoding the variable region of the WN1 222-5 heavy chain from a WN1 222-5 mRNA preparation. The amplified DNA fragment is then sequenced and its V, D and J segments are determined.

Cloning Step No. 2

Another downstream primer complementary to the J segment and BstEII
having the nucleotide sequence 5′ GGAGACGGTGACCGAGGTT 3′ is constructed.

To introduce the BstEII restriction site, the original DNA-sequence as naturally found, is slightly modified.

The J-specific downstream primer and the upstream primer already

used in the cloning step No. 1 are used to amplify a DNA fragment encoding the variable region of the WN1 222-5 heavy chain from a WN1 222-5 cDNA preparation. The amplification of the WN1 58-9 is made analogously to the one of WN1 222-5. The amplified DNA fragment is further cleaved with Sal I and BstE II and cloned into a heavy chain cassette treated with the same enzyme.

The heavy chain cassette is prepared as follows:

A 2.3 kb EcoRI-SalI DNA fragment comprising the promoter and the leader sequence of the gene encoding the heavy chain of the RFT2 antibody (Heinrich et al, J. of Immunol. (1989) 143: 3589) is cloned into the polylinker region of the cloning vector pBluescript II SK- (Stratagene). Downstream from this insertion a 0.4 kb BstEII-BamHI DNA fragment comprising the J segment and the beginning of the major intron of the gene encoding an anti-cytomegalovirus antibody (Newkirk et al, J. Clin. Invest. (1988) 81: 1511).

The EcoRI-BamHI fragment is then transferred into pSV2-neo-Eμ-huCγ1 (Heinrich et al; supra) which contains the human heavy chain enhancer (Eμ) and the sequence encoding the human γ1 constant part.

Example 6 Cloning of the WN1-222-5 or WN1 58-9 Light Chain Variable Region by PCR and Construction of a Chimeric Gene

Cloning steps No. 1 and 2 of Example 5 are repeated using the following primers:

Upstream primer:

MluI

5' AGGT ACG CGT TGT GAC ATC CAG ATG AAC CAG TCT CC 3'

Thr Arg Cys Val Ile Gln Met Asn Gln Ser Pro

Downstream primer specific for the κ constant part:

5' GCACACGACTGAGGCCACCTC 3'

Downstream primer specific for the J segment

HindIII

5′ CGTTTGATTTCAAGCTTGGTG 3′

The amplified DNA fragment is further cleaved with MluI and HindIII and cloned into a light chain cassette treated with the same enzyme. WN1 222-5 and WN1 58-9 are analogously treated.

The light chain cassette is prepared as follows: A 1.3 kb EcoRI-MluI DNA fragment comprising the promoter and the leader sequence of the gene encoding the light chain of the RFT2 antibody (Heinrich et al; Supra) is cloned into the polylinker region of the cloning vector pBluescript II SK⁻ (Stratagene). Downstream from this insertion, a 0.4 kb HindIII-XbaI DNA fragment comprising the J segment and the beginning of the major intron of the gene encoding light chain of RFT2 is cloned.

The EcoRI-XbaI fragment is then transferred into pSV2-DHFR-Eμ-huCκ which is constructed as follows:

A 1.1 kb XbaI - XbaI fragment encoding the murine heavy chain enhancer (Heinrich et al; supra) together with a SphI - HindIII fragment encoding the human κ constant part is subcloned in phage M13 mp18 (Boehringer Mannheim). After disruption of restriction sites by mutagenesis a filled-in EcoRI - HindIII fragment comprising the sequence for the murine heavy chain enhancer (Eμ) and the human κ constant part (huCκ) is cloned in the filled in EcoRI - BamHI site of pSV2-DHFR.

Example 7 Expression of a WN1 222-5 or WN1 58-9 chimeric antibody

The expression vectors as obtained in Examples 5 and 6 are co-transferred in a mouse myeloma cell line SP2/0 (ATCC CRL 1581) by

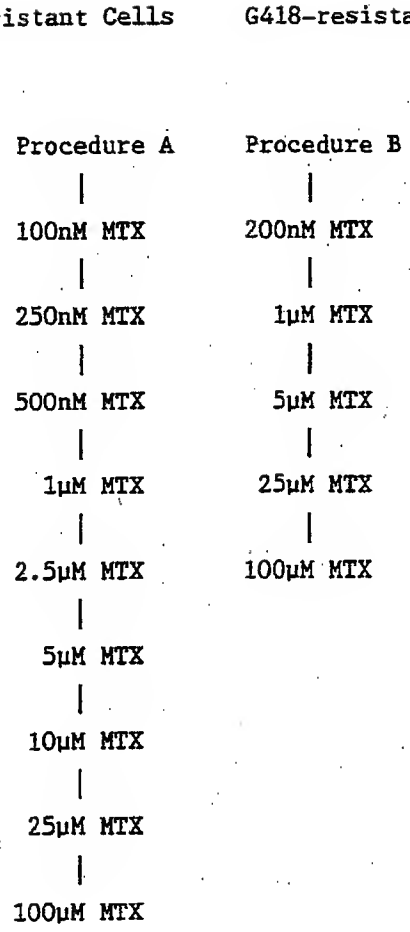
electroporation using a gene pulser apparatus from Bio Rad Laboratories. This technique is known to create stable transfectants at a high frequency. The SP2/0 cell line fails to produce endogeneous heavy and light chains and is sensitive to Gentamycin (G 418) at a concentration of 0.8 mg/l.

SP2/0 cells are grown in the usual growth medium (RPMI + 10% FCS + $5x10^{-5}$ β-mercaptoethanol) harvested in the log phase of growth and washed with the electroporation buffer (Bio-Rad). Cell concentration is adjusted to $2x10^7$ cells/ml. To 0.8 ml of the cell suspension is added 15-20 µg of each plasmid. The mixture is placed on ice and left to stand for 10 min. Then the cells are subjected to an electrical pulse (280 Volt; 25 µF) and again left to stand for 15 min. Cells are transferred to the usual growth medium and incubated at 37°C in a CO_2 incubator.

After 3-day incubation, selection for G 418 resistance is started. Cells are resuspended in fresh medium containing 1.4 mg/ml G 418. The cultures yield growing cells after 10-14 day-incubation in the presence of G 418. After 2-week incubation, the supernatants of the confluent cultures are tested for human IgG expression in a sandwich-type ELISA (anti-human κ-light chain / supernatant /anti-human IgG-alkaline phosphatase conjugate).

This test indicates that complete antibody molecules are secreted in all cultures at varying concentrations in the range of 50-500 ng/ml.

To select cells in which the DHFR gene is amplified and therefore secrete high amounts of the desired antibody two selection procedures for Methotrexate (MTX) resistance are carried out as described below. For this purpose, the G 418 resistant cell pools are each divided and amplification is proceeded either according to procedure A (MTX increase by a factor of 2 or 2.5) or procedure B (MTX increase by a factor of 5).



Each amplification step comprises inoculating the cells at a density of $2x10^5$ cells/ml in the usual growth medium supplemented with G 418 at 1.4 mg/ml and with MTX at the concentration of choice. After 72 hour incubation, cells and the supernatant separated. Antibody secretion is monitored either by ELISA or by HPLC using a protein A column.

Most of the pools reach a maximum of specific antibody production at a certain MTX concentration. The best producing pools are cloned by limiting dilution. Subsequently, the antibody is purified from a culture supernatant by elution on a protein A affinity column.

SEQUENCE IDENTIFIER No.1

Subject matter: The immunoglobulin heavy chain variable domain of the WN1 222-5 antibody

Sequence type: Nucleotide sequence and its corresponding amino acid sequence

Length: 361 nucleotides

Original source: A murine hybridoma

Features of the amino acid sequence:

hFR1 :	from a.a.	1 to 30
hCDR1:	from a.a	31 to 35
hFR2 :	from a.a.	36 to 49
hCDR2:	from a.a.	50 to 67
hFR3 :	from a.a.	68 to 100
hCDR3:	from a.a.	101 to 109
hFR4 :	from a.a.	110 to 120.

```
GAG GTG AAG CTG GTG GAG TCT GGA GGA GGC TTG GTA CAG CCG GGG GGT      48
Glu Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
                  5                  10                  15

TCT CTG AGT CTC TCC TGT GCA GCT TCT GGA TTC ACC TTC AGT GAT TAC      96
Ser Leu Ser Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asp Tyr
             20                  25                  30

TAC ATG ACC TGG GTC CGC CAG GCT CCA GGG AAG GCA CCT GAG TGG TTG     144
Tyr Met Thr Trp Val Arg Gln Ala Pro Gly Lys Ala Pro Glu Trp Leu
         35                  40                  45

GCT TTG ATT AGA AAC AAA CGT AAT GGT GAC ACA GCA GAG TAT AGT GCA     192
Ala Leu Ile Arg Asn Lys Arg Asn Gly Asp Thr Ala Glu Tyr Ser Ala
     50                  55                  60

TCT GTG AAG GGT CGG TTC ACC ATC TCC AGA GAT TAT TCC CGA AGC ATC     240
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Tyr Ser Arg Ser Ile
 65                  70                  75                  80

CTC CAT CTT CAA ATG AAT GCC CTG AGA ACT GAG GAC AGT GCC ACT TAT     288
Leu His Leu Gln Met Asn Ala Leu Arg Thr Glu Asp Ser Ala Thr Tyr
                 85                  90                  95

TAT TGT GTA AGA CAG GGA CGG GGC TAT ACT TTG GAC TAT TGG GGT CAA     336
Tyr Cys Val Arg Gln Gly Arg Gly Tyr Thr Leu Asp Tyr Trp Gly Gln
            100                 105                 110

GGA ACC TCA GTC ACC GTC TCC TCA G                                   361
Gly Thr Ser Val Thr Val Ser Ser
        115                 120
```

SEQUENCE IDENTIFIER No.2

Subject matter: The immunoglobulin heavy chain variable domain of the WN1 58-9 antibody

Sequence type: Nucleotide sequence and its corresponding amino acid sequence

Length: 361 nucleotides

Original source: A murine hybridoma

Features of the amino acid sequence:

$hFR1_r$	: from a.a.	1 to 30
hCDR1	: from a.a.	31 to 35
$hFR2_r$	: from a.a.	36 to 49
hCDR2	: from a.a.	50 to 67
$hFR3_r$	: from a.a.	68 to 100
hCDR3	: from a.a.	101 to 109
hFR4	: from a.a.	110 to 120.

```
GAG GTG AAG CTG GTG GAG TCT GGA GGA GGC TTG GTA CAG CCT GGG GGT      48
Glu Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
                  5                  10                  15

TCT CTG CGT CTC TCC TGT GCA GCT TCT GGA TTC ACC TTC ATT GAT TAC      96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ile Asp Tyr
             20                  25                  30

TAC ATG ACC TGG GTC CGC CAT CCG CCA GGG GAG GCA CCT GAA TGG TTG     144
Tyr Met Thr Trp Val Arg His Pro Pro Gly Glu Ala Pro Glu Trp Leu
         35                  40                  45

GCT TTG ATT AGA AAC TAC CGT AAT GGT GAC ACA GCA GAA TAC AGT GCA     192
Ala Leu Ile Arg Asn Tyr Arg Asn Gly Asp Thr Ala Glu Tyr Ser Ala
     50                  55                  60

TCT GTG AGG GGT CGG TTC ACC ATC TCC AGA GAT GAT TCC CAA AGC ATC     240
Ser Val Arg Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Gln Ser Ile
 65                  70                  75                  80

CTC TAT CTT CAA ATG AAT GCC CTG AGA GCT GAG GAC AGT GCC ACT TAT     288
Leu Tyr Leu Gln Met Asn Ala Leu Arg Ala Glu Asp Ser Ala Thr Tyr
                  85                  90                  95

TAC TGT GTA AGA CAG GGA CGG GGC TAT ACT CTG GAC TAC TGG GGT CAA     336
Tyr Cys Val Arg Gln Gly Arg Gly Tyr Thr Leu Asp Tyr Trp Gly Gln
             100                 105                 110

GGA ACC TCA GTC ACC GTC TCC TCA                                    360
Gly Thr Ser Val Thr Val Ser Ser
         115                 120
```

SEQUENCE IDENTIFIER No.3

Subject matter: The immunoglobulin light chain variable domain of the WN1 222-5 antibody

Sequence type: Nucleotide sequence and its corresponding amino acid sequence

Length: 321 nucleotides

Original source: A murine hybridoma

Features of the amino acid sequence:

1FR1	: from a.a.	1	to	23
1CDR1	: from a.a.	24	to	34
1FR2	: from a.a.	35	to	49
1CDR2	: from a.a.	50	to	56
1FR3	: from a.a.	57	to	88
1CDR3	: from a.a.	89	to	97
1FR4	: from a.a.	98	to	107.

DNA and AA sequence

Light Chain:

```
GAC ATC CAG ATG AAC CAG TCT CCA TCC AGT CTG TCT GCA TCC CTC          45
Asp Ile Gln Met Asn Gln Ser Pro Ser Ser Leu Ser Ala Ser Leu
                 5                  10                  15

GGA GAC ACA ATT TCC ATC ACT TGC CGT GCC AGT CAG AAC ATT AAT          90
Gly Asp Thr Ile Ser Ile Thr Cys Arg Ala Ser Gln Asn Ile Asn
                20                  25                  30

ATT TGG TTA AGC TGG TAT CAG CAA AAA CCA GGA AAT GTT CCT AAA         135
Ile Trp Leu Ser Trp Tyr Gln Gln Lys Pro Gly Asn Val Pro Lys
                35                  40                  45

CTT TTA ATC TAT AAG GCT TCC AAC TTG CAC ACA GGC GTC CCA TCA         180
Leu Leu Ile Tyr Lys Ala Ser Asn Leu His Thr Gly Val Pro Ser
                50                  55                  60

AGG TTT AGT GGC AGT GGA TCT GGA ACA GAT TTC ACA TTA ATC ATC         225
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ile Ile
                65                  70                  75

AGC AGT CTG CAG CCT GAA GAC ATT GCC ACT TAC TAC TGT CTA CAG         270
Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Leu Gln
                80                  85                  90

GGT CAA AGT TAT CCT CGT ACG TTC GGT GGA GGC ACC AAG CTG GAG         315
Gly Gln Ser Tyr Pro Arg Thr Phe Gly Gly Gly Thr Lys Leu Glu
                95                 100                 105

ATC AAA C                                                          322
Ile Lys
   107
```

SEQUENCE IDENTIFIER No.4

Subject matter: The immunoglobulin light chain variable domain of the WN1 58-9 antibody

Sequence type: Nucleotide sequence and its corresponding amino acid sequence

Length: 361 nucleotides

Original source: A murine hybridoma

Features of the amino acid sequence:

$1FR1_r$	: from a.a.	1 to 23
1CDR1	: from a.a.	24 to 34
$1FR2_r$	: from a.a.	35 to 49
1CDR2	: from a.a.	50 to 56
$1FR3_r$	: from a.a.	57 to 88
1CDR3	: from a.a.	89 to 97
1FR4	: from a.a.	98 to 107.

```
GAC ATC CAG ATG AAC CAG TCT CCA TCC AGT CTG TCT GCA TCC CTC          45
Asp Ile Gln Met Asn Gln Ser Pro Ser Ser Leu Ser Ala Ser Leu
                 5                  10                  15

GGA GAC ACA ATT ACC ATC ACT TGC CGT GCC AGA CTG AAC ATT AAT          90
Gly Asp Thr Ile Thr Ile Thr Cys Arg Ala Arg Leu Asn Ile Asn
                20                  25                  30

ATT TGG TTA AGT TGG TAC CAG CAG AAA GCA GGA AAT ATT CCT AAA         135
Ile Trp Leu Ser Trp Tyr Gln Gln Lys Ala Gly Asn Ile Pro Lys
                35                  40                  45

CTT TTG ATC TCT AAG GCT TCC AAC TTG CAC ACA GGC GTC CCA TCA         180
Leu Leu Ile Ser Lys Ala Ser Asn Leu His Thr Gly Val Pro Ser
                50                  55                  60

AGG TTT AGT GGC AGT GGA TCT GGA ACA GAT TTC ACA TTA ACC ATC         225
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

AGC AGT CTG CGG CCT GAA GAC ATT GCC ACT TAC TAC TGT CTA CAG         270
Ser Ser Leu Arg Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Leu Gln
                80                  85                  90

GGT CAA AGT TAT CCT CGT ACG TTC GGT GGA GGC ACC AAG CTT GAA         315
Gly Gln Ser Tyr Pro Arg Thr Phe Gly Gly Gly Thr Lys Leu Glu
                95                 100                 105

ATC AAA                                                            321
Ile Lys
   107
```

Table I

Region	Location on the heavy chains	Location on the light chains
FR1/FR1$_r$	amino acid 1 to 30	amino acid 1 to 23
CDR1/CDR1	amino acid 31 to 35	amino acid 24 to 34
FR2/FR2$_r$	amino acid 36 to 49	amino acid 35 to 49
CDR2/CDR2	amino acid 50 to 67	amino acid 50 to 56
FR3/FR3$_r$	amino acid 68 to 100	amino acid 57 to 88
CDR3	amino acid 101 to 109	amino acid 89 to 97
FR4	amino acid 110 to 120	amino acid 98 to 107

T A B L E II A (first page)

CHEMOTYPE		STRAIN		SUPPLIER	WN1 222-5		
mAb							
					100 ng/ml	10 ng/ml	1 ng/ml
LPS	Smooth	E. coli	02	Univ. Edinburgh	+++++	++++	++
LPS	Smooth	E. coli	04	Univ. Edinburgh	+++++	+++++	++++
LPS	Smooth	E. coli	06	Univ. Edinburgh	+++++	+++++	+++
LPS	Smooth	E. coli	012	Univ. Edinburgh	+++++	+++++	++
LPS	Smooth	E. coli	015	Univ. Edinburgh	+++++	+++++	++++
LPS	Smooth	E. coli	016	Univ. Edinburgh	+++++	+++++	++
LPS	Smooth	E. coli	018K-	Univ. Edinburgh	+++++	+++++	+++
LPS	Smooth	E. coli	018K+	Univ. Edinburgh	+++++	+++++	++++
LPS	Smooth	E. coli	026B6	Difco	+++++	+++++	+++
LPS	Smooth	E. coli	055B5	Difco	+++++	++	+
LPS	Smooth	E. coli	075	Univ. Edinburgh	+++++	+++++	+++
LPS	Smooth	E. coli	086	Univ. Edinburgh	+++++	+++++	+++
LPS	Smooth	E. coli	0111B4	Difco	+++++	+++++	++
LPS	Smooth	E. coli	0127B8	Difco	+++++	+++++	+++
LPS	Smooth	E. coli	0128B12	Difco	++++	+++	++
LPS	Smooth	E. coli	K235	List	+++++	+++++	+++
LPS	Smooth	S. minnesota	wt	List	+++++	+++++	+++
LPS	Smooth	S. typhimurium	wt	Difco	+++++	+++++	++
LPS	Smooth	S. typhimurium	B0 ag 0:4,5,12 (SH 4809)	Bio-Carb	+++++	++++	+
LPS	Smooth	S. typhimurium	B0 ag 0:1,4,5,12 (SL 3622)	Bio-Carb	+++++	++++	+
LPS	Smooth	S. typhimurium	B0 ag 0:4,5,12^2 (SH 4305)	Bio-Carb	++++++	+++++	+
LPS	Smooth	S. typhi	D0 ag 0:9,12^2 (253 Ty)	Bio-Carb	+++++	+++++	+++
LPS	Smooth	S. newport	C2 ag 0:6,8	Bio-Carb	+++++	+++++	+++
LPS	Smooth	S. enteridis	D0 ag 0:9,12 (SH 1262)	Bio-Carb	+++++	+++++	+++
LPS	Smooth	S. thompson	C1 ag 0:6,7, (ls40)	Bio-Carb	+++++	+++++	++++
LPS	Smooth	S. abortus equi	(H1178)	Institut Borstel	+++++	+++++	++++

TABLE II A (second page)

LPS	cCore	E. coli	K12	Univ. Edinburgh	+++++	+++++	++
LPS	cCore	E. coli	C62	Univ. Edinburgh	++++	+++++	++
LPS	cCore	E. coli	R1	Institut Borstel	+++++	+++++	++
LPS	cCore	E. coli	R2	Institut Borstel	+++++	+++++	++
LPS	cCore	E. coli	R3	Institut Borstel	+++++	+++++	++++
LPS	cCore	E. coli	R4	Institut Borstel	+++++	+++++	+++
LPS	cCore	S. minnesota	Ra R60	List	+++++	+++++	+++
LPS	cCore	S. typhimurium	TV119	Sigma	+++++	+++++	+++
LPS	cCore	S. typhimurium	1542	Univ. Edinburgh	+++++	+++++	+++
LPS	cCore	K. aerogenes	M10B	Univ. Edinburgh	-	-	-
LPS	Rb2	S. minnesota	R345	List	++++++	+++++	++++
LPS	Rb3	S. minnesota		Bio-Carb	++++++	+++++	++
LPS	Rc	E. coli	J5	List	++++++	+++++	++++
LPS	Rc	S. typhimurium	878	Univ. Edinburgh	++++++	+++++	++
LPS	Rc	S. typhimurium	SL684	Sigma	++++++	+++++	++++
LPS	Rc	P. aeruginosa	PAC605	Univ. Edinburgh	+	+	+
LPS	RcP-	S. minnesota	R5	List	++++++	+++++	++++
LPS	Rd2	E. coli	F583	Sigma	++++++	+++++	+++
LPS	Rd1P-	S. minnesota	R7	List	+++	++	+
LPS	Rd2	S. minnesota	R4 (V594)	Institut Borstel	++++	+++	++
LPS	Re	E. coli	K12 (D31m4)	List	++++	++++	++
LPS	Re	E. coli	F515	Institut Borstel	+++++	+++++	++++
LPS	Re	S. minnesota	R595	List	+	+	-
LPS	Re	S. typhimurium	SL1102	Univ. Edinburgh	-	-	-
LPS	Re	S. typhimurium	SL1181	Sigma	-	-	-

TABLE II A (third page)

Lipid A	E. coli	K12 (ex-D31m4)	List
Lipid A	S. minnesota	R595	List
BSA			-

Purified native LPS (2 ug/ml) were used to coat the plates
Values are reported as O.D., one + equals 0.5 O.D. (405 nm).

SUBSTITUTE SHEET

T A B L E II B (first page)

CHEMOTYPE			STRAIN	SUPPLIER	WN1 58-9 MAb 100 ng/ml	10ng/ml	1 ng/ml
LPS	Smooth	E. coli	02	Univ. Edinburgh	++++	++++	+
LPS	Smooth	E. coli	04	Univ. Edinburgh	+++++	++++	+
LPS	Smooth	E. coli	06	Univ. Edinburgh	++++	+++++	-
LPS	Smooth	E. coli	012	Univ. Edinburgh	++++	+++++	+
LPS	Smooth	E. coli	015	Univ. Edinburgh	+++++	+++++	++
LPS	Smooth	E. coli	016	Univ. Edinburgh	++++	+++++	+
LPS	Smooth	E. coli	018K-	Univ. Edinburgh	++++	++++	+
LPS	Smooth	E. coli	018K+	Univ. Edinburgh	+++++	+++++	++
LPS	Smooth	E. coli	026B6	Difco	+++++	+++++	+
LPS	Smooth	E. coli	055B5	Difco	+++	+	-
LPS	Smooth	E. coli	075	Univ. Edinburgh	++++	++	-
LPS	Smooth	E. coli	086	Univ. Edinburgh	++++	++++	+
LPS	Smooth	E. coli	0111B4	Difco	++++	++++	+
LPS	Smooth	E. coli	0127B8	Difco	++++	+++	+
LPS	Smooth	E. coli	0128B12	Difco	++	++	-
LPS	Smooth	E. coli	K235	List	++++	++++	+
LPS	Smooth	S. minnesota	wt	List	++++	++++	+
LPS	Smooth	S. typhimurium	wt	Difco	++++	++++	+
LPS	Smooth	S. typhimurium	BO ag 0:4,5,12 (SH 4809)	Bio-Carb	++++	+++	-
LPS	Smooth	S. typhimurium	BO ag 0:1,4,5,12 (SL 3622)	Bio-Carb	++++	+++	+
LPS	Smooth	S. typhimurium	BO ag 0:4,5,12^2 (SH 4305)	Bio-Carb	++++	++++	+
LPS	Smooth	S. typhi	DO ag 0:9, 12^2 (253 Ty)	Bio-Carb	+++++	+++++	+
LPS	Smooth	S. newport	C2 ag 0:6,8	Bio-Carb	+++++	+++++	+
LPS	Smooth	S. enteridis	DO ag 0:9,12 (SH 1262)	Bio-Carb	+++++	++++	+
LPS	Smooth	S. thompson	C1 ag 0:6,7, (1s40)	Bio-Carb	+++++	+++++	++
LPS	Smooth	S. abortus equi	(H1178)	Institut Borstel	+++++	+++++	++
LPS	cCore	E. coli	K12	Univ. Edinburgh	++++	++++	+
LPS	cCore	E. coli	C62	Univ. Edinburgh	+++++	+++++	+++
LPS	cCore	E. coli	R1	Institut Borstel	+++++	+++++	+
LPS	cCore	E. coli	R2	Institut Borstel	++++	+++	+
LPS	cCore	E. coli	R3	Institut Borstel	+++++	+++++	+

T A B L E II B (second page)

LPS	cCore	E. coli	R4	Institut Borstel	++++	+++++	+
LPS	cCore	S. minnesota	Ra R60	List	++++	+++++	+
LPS	cCore	S. typhimurium	TV119	Sigma	++++	++++	+
LPS	cCore	S. typhimurium	1542	Univ. Edinburgh	++++	+++++	+
LPS	cCore	K. aerogenes	M10B	Univ. Edinburgh	-	-	-
LPS	Rb2	S. minnesota	R345	List	+++++	+++++	+++
LPS	Rb3	S. minnesota		Bio-Carb	+++++	+++++	+
LPS	Rc	E. coli	J5	List	+++++	+++++	+
LPS	Rc	S. typhimurium	878	Univ. Edinburgh	+++++	+++++	+
LPS	Rc	P. typhimurium	SL684	Sigma	+++++	+++++	++
LPS	Rc	P. aeruginosa	PAC605	Univ. Edinburgh	+	+	-
LPS	RcP⁻	S. minnesota	R5	List	+++++	+++++	++
LPS	Rd2	E. coli	F583	Sigma	+++++	+++++	++
LPS	Rd1P⁻	S. minnesota	R7	List	++++	++	-
LPS	Rd2	S. minnesota	R4 (V594)	Institut Borstel	++++	+++	-
LPS	Re	E. coli	K12 (D31m4)	List	+++++	+++++	++
LPS	Re	E. coli	F515	Institut Borstel	+++++	+++++	+++
LPS	Re	S. minnesota	R595	List	+	-	-
LPS	Re	S. typhimurium	SL1102	Univ. Edinburgh	-	-	-
LPS	Re	S. typhimurium	SL1181	Sigma	-	-	-
Lipid A		E. coli	K12 (ex-D31m4)	List	-	-	-
Lipid A		S. minnesota	R595	List	-	-	-
BSA					-	-	-

Purfied native LPS (2 µg/ml) were used to coat the plates
Values are reported as O.D., one + equals 0.5 O.D. (405 nm).
BSA = bovine serum albumin

T A B L E II C (first page)

CHEMOTYPE			STRAIN	SUPPLIER	H1 61-2 IgG1			
					1µg/ml	100 ng/ml	10ng/ml	1 ng/ml
LPS	Smooth	E. coli	02	Univ. Edinburgh	++++	+++++	++	-
LPS	Smooth	E. coli	06	Univ. Edinburgh	+++	+++++	++	-
LPS	Smooth	E. coli	012	Univ. Edinburgh	+++++	+++++	+++++	+++
LPS	Smooth	E. coli	015	Univ. Edinburgh	+++++	+++++	++++	+
LPS	Smooth	E. coli	016	Univ. Edinburgh	+	++	-	-
LPS	Smooth	E. coli	018K-	Univ. Edinburgh	+++++	+++++	+++++	+
LPS	Smooth	E. coli	018K+	Univ. Edinburgh	+++++	+++++	+++++	++
LPS	Smooth	E. coli	026B6	Difco	+++++	+++++	+++++	+
LPS	Smooth	E. coli	055B5	Difco	+++	++++	-	-
LPS	Smooth	E. coli	075	Univ. Edinburgh	+++	+++++	+++	-
LPS	Smooth	E. coli	086	Univ. Edinburgh	+++++	+++++	++++	+
LPS	Smooth	E. coli	0111B4	Difco	+++++	+++++	+++++	++
LPS	Smooth	E. coli	0127B8	Difco	+++++	+++++	+++++	+++
LPS	Smooth	E. coli	0128B12	Difco	-	-	-	-
LPS	Smooth	E. coli	K235	List	++++	+++++	+++	-
LPS	Smooth	S. minnesota	wt	List	++++	++++	++	-
LPS	Smooth	S. typhimurium	wt	Difco	+++++	+++++	+++++	+
LPS	cCore	E. coli	K12	Univ. Edinburgh	+++++	+++++	+++++	+++
LPS	cCore	E. coli	C62	Univ. Edinburgh	++++++	+++++	+++++	++
LPS	cCore	E. coli	R1	Institut Borstel	++++++	+++++	+++++	+++
LPS	cCore	E. coli	R2	Institut Borstel	+++++	+++++	+++++	++++
LPS	cCore	E. coli	R3	Institut Borstel	+++++	+++++	+++++	++
LPS	cCore	E. coli	R4	Institut Borstel	++++++	+++++	+++++	++++

T A B L E II C (second page)

LPS	cCore	S. minnesota	Ra R60	List	++++++	+++++	+++++	++++
LPS	cCore	S. typhimurium	TV119	Sigma	++++++	+++++	+++++	+
LPS	cCore	S. typhimurium	1542	Univ. Edinburgh	+++++	+++++	+++++	++
LPS	cCore	K. aerogenes	M10B	Univ. Edinburgh	-	-	-	-
LPS	Rb2	S. minnesota	R345	List	++++++	+++++	++	-
LPS	Rc	E. coli	J5	List	++++++	+++++	+++++	++++
LPS	Rc	S. typhimurium	878	Univ. Edinburgh	++++++	++++++	+++++	++++
LPS	Rc	P. typhimurium	SL684	Sigma	+++++	+++++	+++++	++++
LPS	Rc	P. aeruginosa	PAC605	Univ. Edinburgh	-	-	-	-
LPS	RcP⁻	S. minnesota	R5	List	++++++	+++++	+++++	+++
LPS	Rd2	E. coli	F583	Sigma	+++	++	-	-
LPS	Rd1P⁻	S. minnesota	R7	List	-	-	-	-
LPS	Rd2	S. minnesota	R4	Institut Borstel	-	-	-	-
LPS	Re	E. coli	K12 (D31m4)	List	-	-	-	-
LPS	Re	E. coli	F515	Institut Borstel	-	-	-	-
LPS	Re	S. minnesota	R595	List	-	-	-	-
LPS	Re	S. typhimurium	SL1102	Univ. Edinburgh	-	-	-	-
LPS	Re	S. typhimurium	SL1181	Sigma	-	-	-	-
Lipid A		E. coli	K12 (ex-D31m4)		-	-	-	-
Lipid A		S. minnesota	R595		+	++	-	-
BSA					-	-	-	-

Purfied native LPS (2 µg/ml) were used to coat the plates
Values are reported as O.D., one + equals 0.5 O.D. (405 nm).
BSA = bovine serum albumin

T A B L E II D (first page)

CHEMOTYPE			STRAIN	SUPPLIER	SZ 27 19.16.7			
					1µg/ml	100 ng/ml	10ng/ml	1 ng/ml
LPS	Smooth	E. coli	02	Univ. Edinburgh	++	+	-	-
LPS	Smooth	E. coli	06	Univ. Edinburgh	-	-	-	-
LPS	Smooth	E. coli	012	Univ. Edinburgh	+++++	++++++	++++	+
LPS	Smooth	E. coli	015	Univ. Edinburgh	+	+++	++	-
LPS	Smooth	E. coli	016	Univ. Edinburgh	-	-	-	-
LPS	Smooth	E. coli	018K-	Univ. Edinburgh	+	+++	-	-
LPS	Smooth	E. coli	018K+	Univ. Edinburgh	++++	++++	++	-
LPS	Smooth	E. coli	026B6	Difco	+++++	+++++	+++	-
LPS	Smooth	E. coli	055B5	Difco	-	-	-	-
LPS	Smooth	E. coli	075	Univ. Edinburgh	+	+	-	-
LPS	Smooth	E. coli	086	Univ. Edinburgh	++	++++	+++	-
LPS	Smooth	E. coli	0111B4	Difco	+++++	+++++	+++++	+
LPS	Smooth	E. coli	0127B8	Difco	++++++	+++++	+++++	+
LPS	Smooth	E. coli	0128B12	Difco	-	-	-	-
LPS	Smooth	E. coli	K235	List	+++	++	++	-
LPS	Smooth	S. minnesota	wt	List	++	-	-	-
LPS	Smooth	S. typhimurium	wt	Difco	+++++	+++++	+++	-
LPS	cCore	E. coli	K12	Univ. Edinburgh	+++++	+++++	+++++	+
LPS	cCore	E. coli	C62	Univ. Edinburgh	+++++	+++++	+++++	+
LPS	cCore	E. coli	R1	Institut Borstel	+++++	+++++	+++++	+
LPS	cCore	E. coli	R2	Institut Borstel	++++++	+++++	+++++	+
LPS	cCore	E. coli	R3	Institut Borstel	+++++	+++++	+++++	+
LPS	cCore	E. coli	R4	Institut Borstel	+++++	+++++	+++++	+

T A B L E II D (second page)

LPS	cCore	S. minnesota	Ra R60	List	+++++	+++++	+++++	+
LPS	cCore	S. typhimurium	TV119	Sigma	+++++	+++++	++	-
LPS	cCore	S. typhimurium	1542	Univ. Edinburgh	+++++	+++++	++++	+
LPS	cCore	K. aerogenes	M10B	Univ. Edinburgh	-	-	-	-
LPS	Rb2	S. minnesota	R345	List	+++++	++++	++	-
LPS	Rc	E. coli	J5	List	+++++	+++++	+++++	+
LPS	Rc	S. typhimurium	878	Univ. Edinburgh	+++++	+++++	+++++	+
LPS	Rc	P. typhimurium	SL684	Sigma	++++++	+++++	+++++	+
LPS	Rc	P. aeruginosa	PAC605	Univ. Edinburgh	+	-	-	-
LPS	RcP$^-$	S. minnesota	R5	List	+++++	+++++	++++	+
LPS	Rd2	E. coli	F583	Sigma	-	-	-	-
LPS	Rd1P$^-$	S. minnesota	R7	List	-	-	-	-
LPS	Rd2	S. minnesota	R4	Institut Borstel	-	-	+	+
LPS	Re	E. coli	K12 (D31m4)	List	-	-	-	-
LPS	Re	E. coli	F515	Institut Borstel	-	-	-	-
LPS	Re	S. minnesota	R595	List	-	-	-	-
LPS	Re	S. typhimurium	SL1102	Univ. Edinburgh	-	-	-	-
LPS	Re	S. typhimurium	SL1181	Sigma	-	-	-	-
Lipid A		E. coli	K12 (ex-D31m4)	List	-	-	-	-
Lipid A		S. minnesota	R595	List	-	-	-	-
BSA					-	-	-	-

Purfied native LPS (2 µg/ml) were used to coat the plates
Values are reported as O.D., one + equals 0.5 O.D. (405 nm).
BSA = bovine serum albumin

T A B L E III A (first page)

CHEMOTYPE		STRAIN	SUPPLIER	WN1 222-5 100 ng/ml
Bacteria smooth	E. coli	01	Univ. Edinburgh	+++++
Bacteria smooth	E. coli	04	Univ. Edinburgh	+++++
Bacteria smooth	E. coli	06	Univ. Edinburgh	+++++
Bacteria smooth	E. coli	015	Univ. Edinburgh	+++++
Bacteria smooth	E. coli	016	Univ. Edinburgh	+++++
Bacteria smooth	E. coli	018K-	Univ. Edinburgh	+++++
Bacteria smooth	E. coli	075	Univ. Edinburgh	
Bacteria cCore	E. coli	K12	Univ. Edinburgh	+++++
Bacteria cCore	E. coli	C62	Univ. Edinburgh	+++++
Bacteria cCore	E. coli	R1	Institut Borstel	+++++
Bacteria cCore	E. coli	R2	Institut Borstel	+++++
Bacteria cCore	E. coli	R3	Institut Borstel	+++++
Bacteria cCore	E. coli	R4	Institut Borstel	+++++
Bacteria cCore	S. minnesota	Ra R60	Univ. Edinburgh	+++++
Bacteria cCore	S. typhimurium	1135	Univ. Edinburgh	+++++
Bacteria cCore	S. typhimurium	1542	Univ. Edinburgh	+++++
Bacteria cCore	K. aerogenes	M10B	Univ. Edinburgh	-
Bacteria Rb	S. minnesota	R345	Univ. Edinburgh	+++++
Bacteria Rc	E. coli	J5	Univ. Edinburgh	+++++
Bacteria Rc	S. typhimurium	878	Univ. Edinburgh	+++++
Bacteria Rc	P. aeruginosa	PAC 605	Univ. Edinburgh	-
Bacteria Rc	S. minnesota	R5	Univ. Edinburgh	+++++
Bacteria Rd1P-	S. minnesota	R7	Univ. Edinburgh	+++++
Bacteria Rd2	S. minnesota	R4	Univ. Edinburgh	-

TABLE III A (second page)

Bacteria Re	E. coli	F515	Institut Borstel	-
Bacteria Re	S. minnesota	R595	Univ. Edinburgh	-
Bacteria Re	S. typhimurium	SL1102	Univ. Edinburgh	+
BSA				-

Heat killed bacteria (0.5×10^8 cell/ml) were used to coat the plates
Values are reported as O.D., one + equals 0.5 O.D. (405 nm).

SUBSTITUTE SHEET

T A B L E III B (first page)

CHEMOTYPE		STRAIN	SUPPLIER	WN1 58-9 100 ng/ml
Bacteria smooth	E. coli	01	Univ. Edinburgh	+++++
Bacteria smooth	E. coli	04	Univ. Edinburgh	+++++
Bacteria smooth	E. coli	06	Univ. Edinburgh	+++++
Bacteria smooth	E. coli	015	Univ. Edinburgh	+++++
Bacteria smooth	E. coli	016	Univ. Edinburgh	+++++
Bacteria smooth	E. coli	018K-	Univ. Edinburgh	+++++
Bacteria smooth	E. coli	075	Univ. Edinburgh	+++++
Bacteria cCore	E. coli	K12	Univ. Edinburgh	+++++
Bacteria cCore	E. coli	C62	Univ. Edinburgh	+++++
Bacteria cCore	E. coli	R1	Institut Borstel	+++++
Bacteria cCore	E. coli	R2	Institut Borstel	+++++
Bacteria cCore	E. coli	R3	Institut Borstel	+++++
Bacteria cCore	E. coli	R4	Institut Borstel	++++
Bacteria cCore	S. minnesota	Ra R60	Univ. Edinburgh	+++++
Bacteria cCore	S. typhimurium	1135	Univ. Edinburgh	+++++
Bacteria cCore	S. typhimurium	1542	Univ. Edinburgh	+++++
Bacteria cCore	K. aerogenes	M10B	Univ. Edinburgh	-
Bacteria Rb	S. minnesota	R345	Univ. Edinburgh	++++
Bacteria Rc	E. coli	J5	Univ. Edinburgh	+++++
Bacteria Rc	S. typhimurium	878	Univ. Edinburgh	+++++
Bacteria Rc	P. aeruginosa	PAC 605	Univ. Edinburgh	-
Bacteria Rc	S. minnesota	R5	Univ. Edinburgh	+++++
Bacteria Rd1P-	S. minnesota	R7	Univ. Edinburgh	+++++
Bacteria Rd2	S. minnesota	R4	Univ. Edinburgh	-

TABLE III B (second page)

Bacteria Re	E. coli	F515	Institut Borstel	-
Bacteria Re	S. minnesota	R595	Univ. Edinburgh	-
Bacteria Re	S. typhimurium	SL1102	Univ. Edinburgh	+
BSA				-

Heat killed bacteria (0.5×10^8 cell/ml) were used to coat the plates
Values are reported as O.D., one + equals 0.5 O.D. (405 nm).

T A B L E III C (first page)

CHEMOTYPE		STRAIN	SUPPLIER	H1 61-2 IgG1			
				1µg/ml	100 ng/ml	10ng/ml	1ng/nl
Bacteria smooth	E. coli	01	Univ. Edinburgh	+++++	+++++	+++++	+++
Bacteria smooth	E. coli	04	Univ. Edinburgh	+++++	+++++	+++++	++
Bacteria smooth	E. coli	06	Univ. Edinburgh	+++++	+++++	+++++	++
Bacteria smooth	E. coli	015	Univ. Edinburgh	+++++	+++++	+++++	+
Bacteria smooth	E. coli	016	Univ. Edinburgh	+++++	+++++	+++++	+++
Bacteria smooth	E. coli	018K-	Univ. Edinburgh	+	-	-	-
Bacteria smooth	E. coli	075	Univ. Edinburgh	+++++	+++++	+++++	++++
Bacteria cCore	E. coli	K12	Univ. Edinburgh	+++++	+++++	+++++	+++
Bacteria cCore	E. coli	C62	Univ. Edinburgh	+++++	+++++	+++++	++++
Bacteria cCore	E. coli	R1	Institut Borstel	+++++	+++++	+++++	+++
Bacteria cCore	E. coli	R2	Institut Borstel	+++++	+++++	+++++	++++
Bacteria cCore	E. coli	R3	Institut Borstel	+++++	+++++	+++++	++++
Bacteria cCore	E. coli	R4	Institut Borstel	+++++	+++++	+++++	++++
Bacteria cCore	S. minnesota	Ra R60	Univ. Edinburgh	++++++	+++++	+++++	+++
Bacteria cCore	S. typhimurium	1135	Univ. Edinburgh	+++++	+++++	+++++	++++
Bacteria cCore	S. typhimurium	1542	Univ. Edinburgh	+++++	+++++	+++++	++++
Bacteria cCore	K. aerogenes	M10B	Univ. Edinburgh	-	-	-	-
Bacteria Rb	S. minnesota	R345	Univ. Edinburgh	++	+	-	-
Bacteria Rc	E. coli	J5	Univ. Edinburgh	+++++	+++++	+++++	++
Bacteria Rc	S. typhimurium	878	Univ. Edinburgh	+++++	+++++	+++++	+++
Bacteria Rc	P. aeruginosa	PAC 605	Univ. Edinburgh	-	-	-	-
Bacteria Rc	S. minnesota	R5	Univ. Edinburgh	+++++	+++++	+++++	++
Bacteria Rd1P-	S. minnesota	R7	Univ. Edinburgh	+++++	++++	++	+
Bacteria Rd2	S. minnesota	R4	Univ. Edinburgh	-	-	-	-

TABLE III C (second page)

Bacteria Re	E. coli	F515	Institut Borstel	-
Bacteria Re	S. minnesota	R595	Univ. Edinburgh	-
Bacteria Re	S. typhimurium	SL1102	Univ. Edinburgh	-
BSA				-

Heat killed bacteria (0.5×10^8 cell/ml) were used to coat the plates
Values are reported as O.D., one + equals 0.5 O.D. (405 nm).

T A B L E III D (first page)

CHEMOTYPE		STRAIN	SUPPLIER	SZ 27 19.16.7			
				1µg/ml	100 ng/ml	10ng/ml	1ng/nl
Bacteria smooth	E. coli	01	Univ. Edinburgh	++++++	+++++	+++++	++
Bacteria smooth	E. coli	04	Univ. Edinburgh	++++++	+++++	+++++	++
Bacteria smooth	E. coli	06	Univ. Edinburgh	+++++	+++++	+++++	++
Bacteria smooth	E. coli	015	Univ. Edinburgh	++++++	+++++	+++++	+
Bacteria smooth	E. coli	016	Univ. Edinburgh	++++++	+++++	+++++	++
Bacteria smooth	E. coli	018K-	Univ. Edinburgh	++++	+++	+	-
Bacteria smooth	E. coli	075	Univ. Edinburgh	+++++	++++	+++++	+
Bacteria cCore	E. coli	K12	Univ. Edinburgh	+++++	+++++	+++++	++
Bacteria cCore	E. coli	C62	Univ. Edinburgh	+++++	+++++	+++++	++
Bacteria cCore	E. coli	R1	Institut Borstel	+++++	+++++	+++++	+
Bacteria cCore	E. coli	R2	Institut Borstel	+++++	+++++	+++++	+
Bacteria cCore	E. coli	R3	Institut Borstel	+++++	+++++	+++++	+
Bacteria cCore	E. coli	R4	Institut Borstel	+++++	+++++	+++++	++
Bacteria cCore	S. minnesota	Ra R60	Univ. Edinburgh	+++++	+++++	+++++	+
Bacteria cCore	S. typhimurium	1135	Univ. Edinburgh	++++++	+++++	+++++	+
Bacteria cCore	S. typhimurium	1542	Univ. Edinburgh	++++++	+++++	+++++	++
Bacteria cCore	K. aerogenes	M10B	Univ. Edinburgh	-	-	-	-
Bacteria Rb	S. minnesota	R345	Univ. Edinburgh	+	-	-	-
Bacteria Rc	E. coli	J5	Univ. Edinburgh	++++++	+++++	+++++	+
Bacteria Rc	S. typhimurium	878	Univ. Edinburgh	++++++	+++++	+++++	+
Bacteria Rc	P. aeruginosa	PAC 605	Univ. Edinburgh	-	-	-	-
Bacteria Rc	S. minnesota	R5	Univ. Edinburgh	++++++	+++++	+++++	+
Bacteria Rd1P-	S. minnesota	R7	Univ. Edinburgh	+++++	++++	++	-
Bacteria Rd2	S. minnesota	R4	Univ. Edinburgh	-	-	-	-

TABLE III D (second page)

Bacteria Re	E. coli	F515	Institut Borstel	-		-		-		-	
Bacteria Re	S. minnesota	R595	Univ. Edinburgh	-		-		-		-	
Bacteria Re	S. typhimurium	SL1102	Univ. Edinburgh	-		-		-		-	
BSA				-		-					

Heat killed bacteria (0.5×10^8 cell/ml) were used to coat the plates
Values are reported as O.D., one + equals 0.5 O.D. (405 nm).

CLAIMS

1. A monoclonal antibody which recognizes an epitope in the core region of the LPS molecule and which is cross-protective against endotoxemia caused by at least two different Gram-negative bacterial strains having different core structures.

2. A monoclonal antibody according to Claim 1 which recognizes an epitope which is completely present in the Rc core structure of E. coli and is also present in the complete core.

3. A monoclonal antibody according to Claim 1 or Claim 2 which is murine.

4. A monoclonal antibody according to any one of the preceding claims which is of IgG isotype.

5. A hybridoma cell line producing a monoclonal antibody according to any one of the preceding claims.

6. A method for the production of a monoclonal antibody according to any one of claims 1 to 4 characterized by the steps of

a) Immunizing an animal with a plurality of types of LPS molecule

b) Fusing spleen cells from the animal with an immortalizing cell line to produce hybridomas

c) Screening the hybridomas to select those producing cross-reactive antibodies

d) Further screening the hybridomas to select those producing protective antibodies

and e) Growing the selected hybridoma and isolating the antibody produced.

7. A method according to claim 6 in which the animal is immunized with a

cocktail of different rough strains of heat-killed Gram-negative bacteria.

8. A method according to claim 6 in which the animal is immunized sequentially with a number of different rough strains of heat-killed Gram- negative bacteria, only one strain being administered at any one time.

9. A method according to any one of claims 6 to 8 which comprises the additional step:

a') Carrying out an initial screening on the serum of the immunized animal to test the strength and plurality of its immune response, selecting animals with a strong response, and giving such animals a booster immunization before removing its spleen cells.

10. A method according to any one of claims 6 to 9 in which the screening step c) is carried out in an ELISA assay using a series of mixtures of different smooth and rough LPS types.

11. An LPS binding molecule which comprises at least one antigen binding site comprising at least one domain which comprises in sequence, the hypervariable regions hCDR1, hCDR2 and hCDR3 ;
said hCDR1 having the amino acid sequence Asp Tyr Tyr Met Thr;
said hCDR2 having the amino acid sequence Leu Ile Arg Asn W Arg Asn Gly Asp Thr Ala Glu Tyr Ser Ala Ser Val X;

wherein W is Lys or Tyr and X is Lys or Arg;

said hCDR3 having the amino acid sequence Gln Gly Arg Gly Tyr Thr Leu Asp Tyr;
and direct equivalents thereof.

12. A single domain antibody according to Claim 11 comprising in sequence the hypervariable regions hCDR1, hCDR2 and hCDR3 associated with murine or human heavy chain framework regions so as to form an isolated heavy chain variable domain.

13. An LPS binding molecule according to Claim 11 comprising at least one antigen binding site comprising:

a) a first domain comprising in sequence the hypervariable regions hCDR1, hCDR2 and hCDR3 as defined in claim 11 and,

b) a second domain comprising in sequence the hypervariable regions lCDR1, lCDR2 and CDR3;

said lCDR1 having the amino acid sequence Arg Ala Y Z Asn Ile Asn Ile Trp Leu Ser,

wherein Y is Ser or Arg and Z is Gln or Leu;

said lCDR2 having the amino acid sequence Lys Ala Ser Asn Leu His Thr;

said lCDR3 having the amino acid sequence Leu Gln Gly Gln Ser Tyr Pro Arg Thr;

and direct equivalents thereof.

14. An LPS binding molecule according to Claim 13 in which the hypervariable regions are associated with murine or human framework regions.

15. An LPS binding molecule according to Claim 13 or Claim 14 in which the first and the second domains are part of a single common peptide chain.

16. A single chain antibody according to Claim 15 in which the first and the second domains are respectively an Ig heavy chain variable domain and an Ig light chain variable domain, and are covalently bound by a peptide linker consisting of from 10 to 30 amino acids.

17. An LPS binding molecule according to Claim 13 or Claim 14 in which the first domain is part of a heavy chain of at least a fragment of an Ig molecule, and the second domain is part of a light chain of at least a fragment of an Ig molecule.

18. An LPS binding molecule according to Claim 17 which is a complete

Ig molecule.

19. An Ig molecule according to Claim 18 which is of IgG isotype.

20. An Ig molecule according to Claim 18 or Claim 19 which is murine.

21. An Ig molecule according to Claim 18 or Claim 19 in which the variable domains are murine and the constant domains are human.

22. An Ig molecule according to Claim 18 or Claim 19 in which the framework regions and the constant domains are human.

23. An Ig molecule according to Claim 20 or Claim 21 in which the heavy chain variable domain has an amino acid sequence substantially identical to that given in Seq. Id. No 1 or alternatively in Seq. Id. No 2 and the light chain variable domain has an amino acid sequence substantially identical to that given in Seq. Id. No 3 or alternatively in Seq. Id. No 4.

24. An Ig molecule according to Claim 23 as dependent on Claim 21, in which the heavy chain constant domain is of human type γ_1 and the light chain constant domain is of human type κ.

25. A DNA construct coding for an amino acid sequence comprising in sequence the hypervariable regions hCDR1, hCDR2 and hCDR3 stated in Claim 11.

26. A DNA construct encoding a heavy chain or fragment thereof and comprising

a) a first part which encodes a variable domain comprising alternately framework and hypervariable regions, said hypervariable regions being in sequence hCDR1, hCDR2 and hCDR3, the amino acid sequences of which are stated in Claim 11; this first part starting with a codon encoding the first amino acid of the variable domain and

ending with a codon encoding the last amino acid of the variable domain, and

b) a second part encoding a heavy chain constant part or fragment thereof which starts with a codon encoding the first amino acid of the constant part of the heavy chain and ends with a codon encoding the last amino acid of the constant part or fragment thereof, followed by a non-sense codon.

27. A DNA construct according to Claim 26 in which the first part encodes a variable domain having an amino acid sequence substantially identical to the amino acid sequence as shown in Seq. Id. No 1 or alternatively No.2 and the second part encodes the constant part of the human γ_1 chain.

28. A DNA construct coding for an amino acid sequence comprising in sequence the hypervariable regions lCDR1, lCDR2 and lCDR3 stated in Claim 13.

29. A DNA construct encoding a light chain or fragment thereof and comprising

a) a first part which encodes a variable domain comprising alternately framework and hypervariable regions; said hypervariable regions being in sequence lCDR1, lCDR2 and lCDR3, the amino acid sequences of which are shown in Seq. Id. No. 3 or in Seq. Id. No 4; this first part starting with a codon encoding the first amino acid of the variable domain and ending with a codon encoding the last amino acid of the variable domain, and

b) a second part encoding a light chain constant part or fragment thereof which starts with a codon encoding the first amino acid of the constant part of the light chain and ends with a codon encoding the last amino acid of the constant part or fragment thereof followed by a non-sense codon.

30. A DNA construct according to Claim 29 in which the first part encodes a variable domain having an amino acid sequence substantially identical to the amino acid sequence as shown in Seq. Id. No 3 or in Seq. Id. No 4 and the second part encodes the constant part of the human κ chain.

31. An expression vector comprising a DNA construct according to any one of claims 25-30 in operative association with a suitable promoter.

32. A host cell transformed with a vector according to Claim 31.

33. A process for the preparation of an LPS binding molecule according to any one of Claims 11-24 comprising the steps of culturing the host cell of Claim 32 and isolating the expressed protein.

34. The use of an LPS binding molecule according to any one of Claims 11-24 as a medicament or diagnostic aid.

35. A pharmaceutical composition comprising an LPS binding molecule according to any one of Claims 11-24 in association with a pharmaceutically acceptable diluent or carrier.

FIG. 1

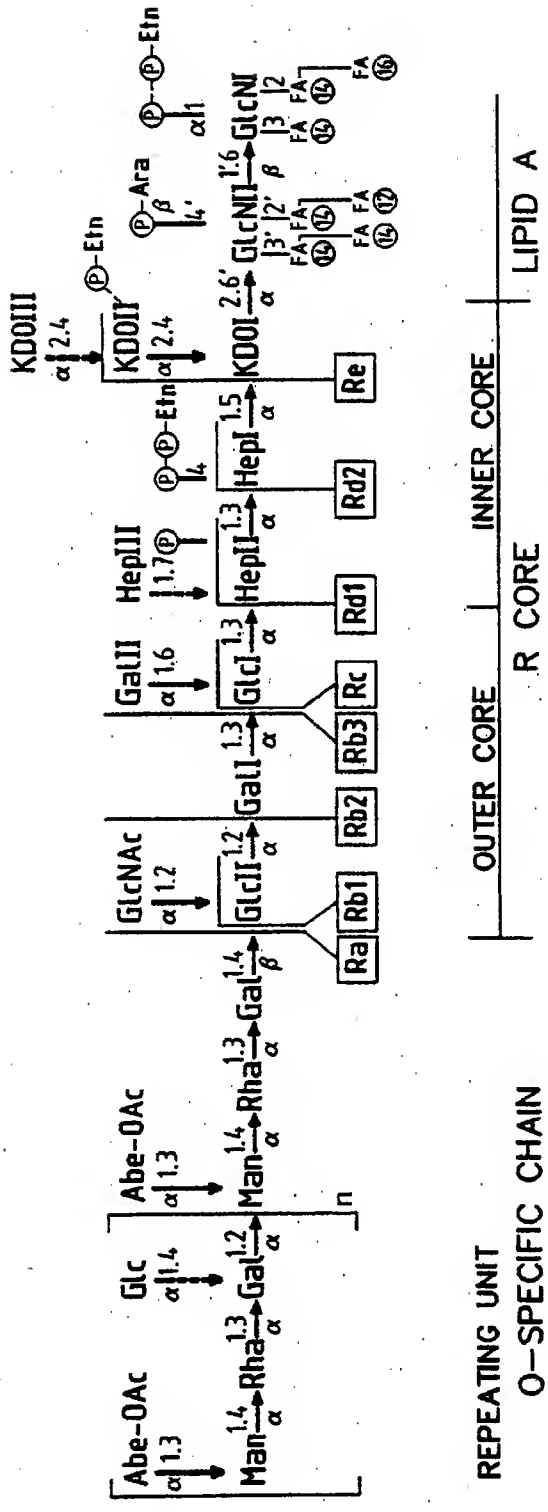

KDOIII
KDOII
KDOI
GalII
HepIII
GlcII
HepII
HepI
GlcNAc
Abe-OAc
Glc
Man
Rha
Gal
Ara
Etn
GlcNI
FA
Ra
Rb1
Rb2
Rb3
Rc
Rd1
Rd2
Re
REPEATING UNIT
O-SPECIFIC CHAIN
OUTER CORE
INNER CORE
R CORE
LIPID A

FIG. 2

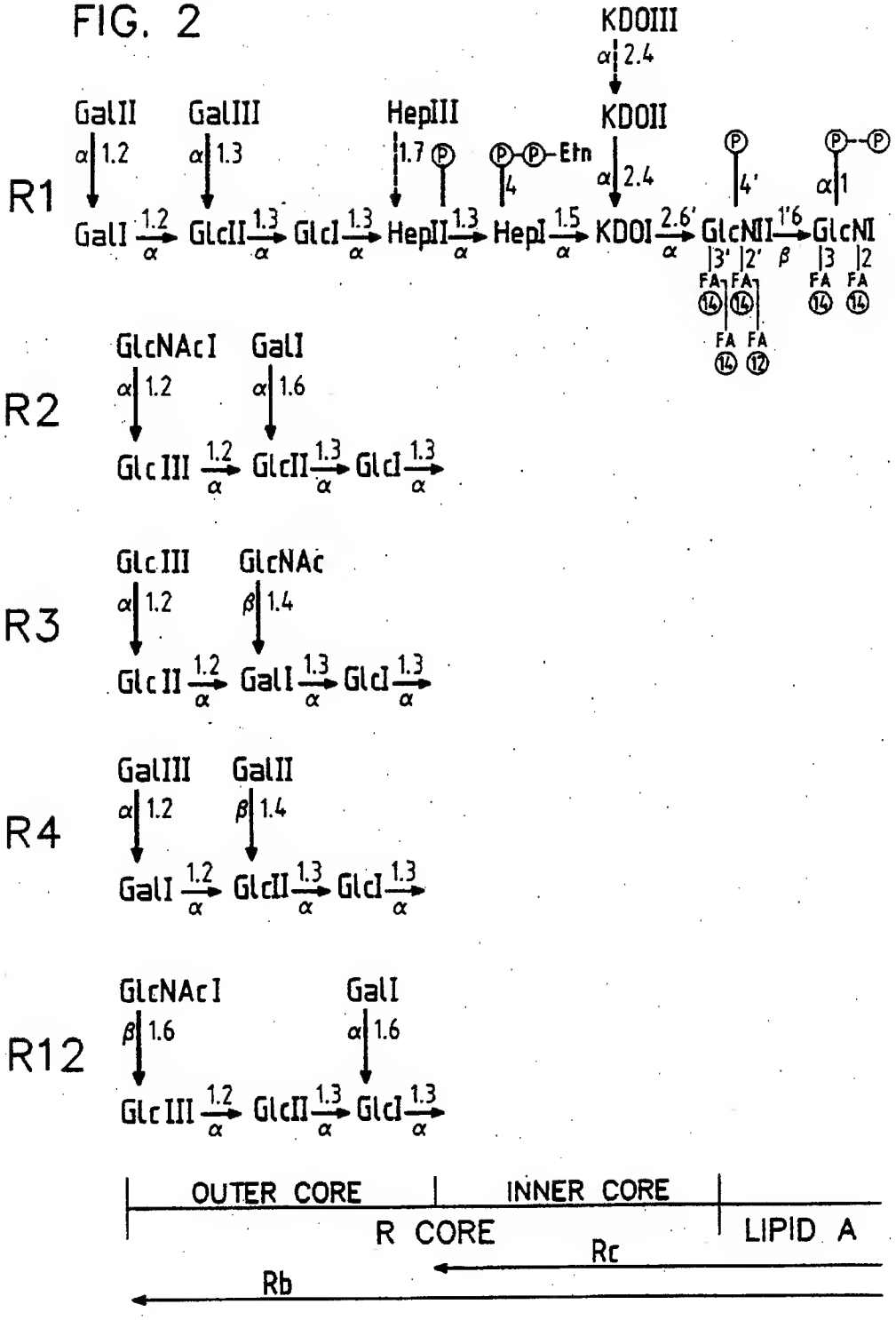

R1
GalII
GalIII
HepIII
KDOIII
KDOII
GalI
GlcII
GlcI
HepII
HepI
KDOI
GlcNII
GlcNI
Etn
R2
GlcNAcI
GalI
GlcIII
R3
GlcNAc
R4
R12
OUTER CORE
INNER CORE
R CORE
LIPID A
Rc
Rb

FIG. 3a

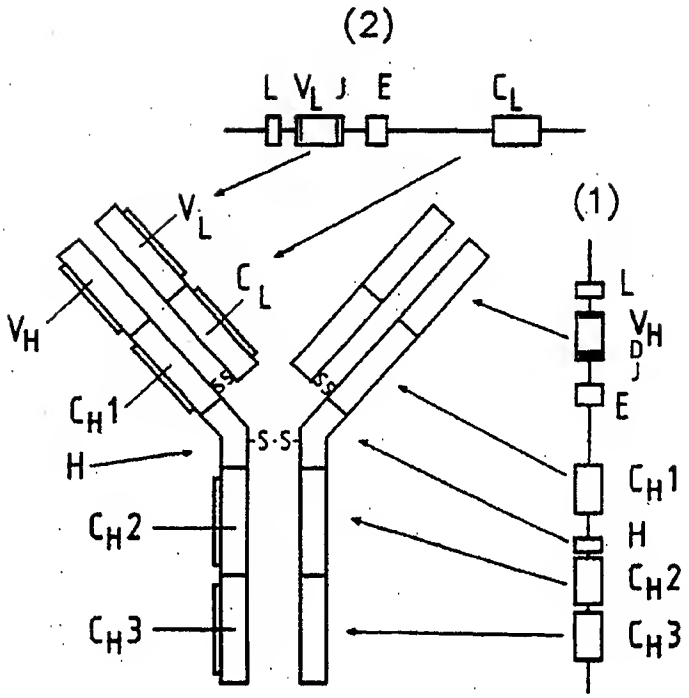

(2)
L
V_L
J
E
C_L
(1)
V_L
C_L
V_H
C_H1
H
C_H2
C_H3
S-S
L
V_H
D
J
E
C_H1
H
C_H2
C_H3

FIG. 3b

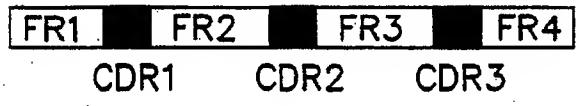

FR1
FR2
FR3
FR4
CDR1
CDR2
CDR3

FIG. 4a

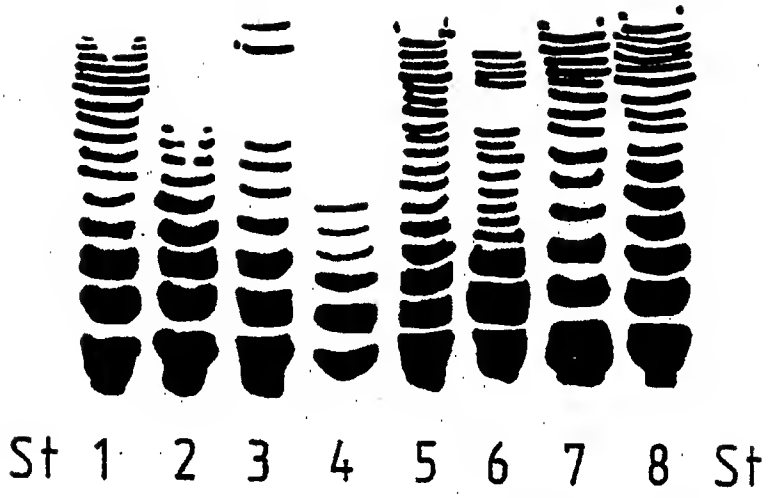


FIG. 4b

FIG. 4c

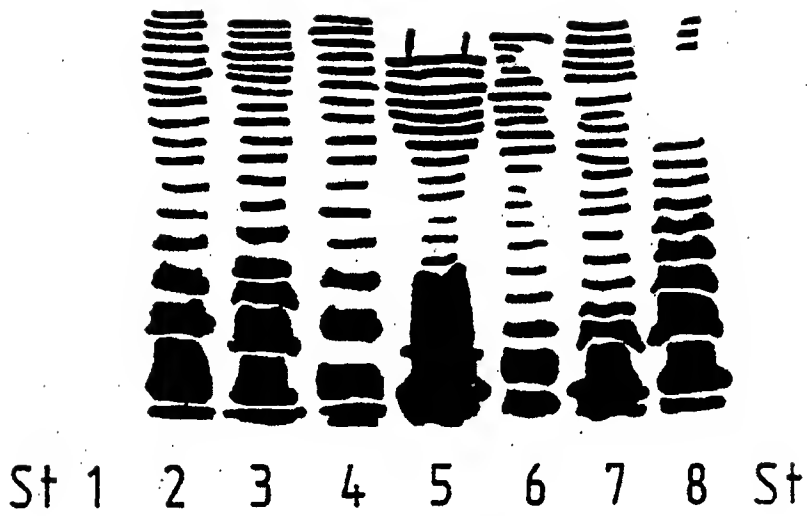


FIG. 4d

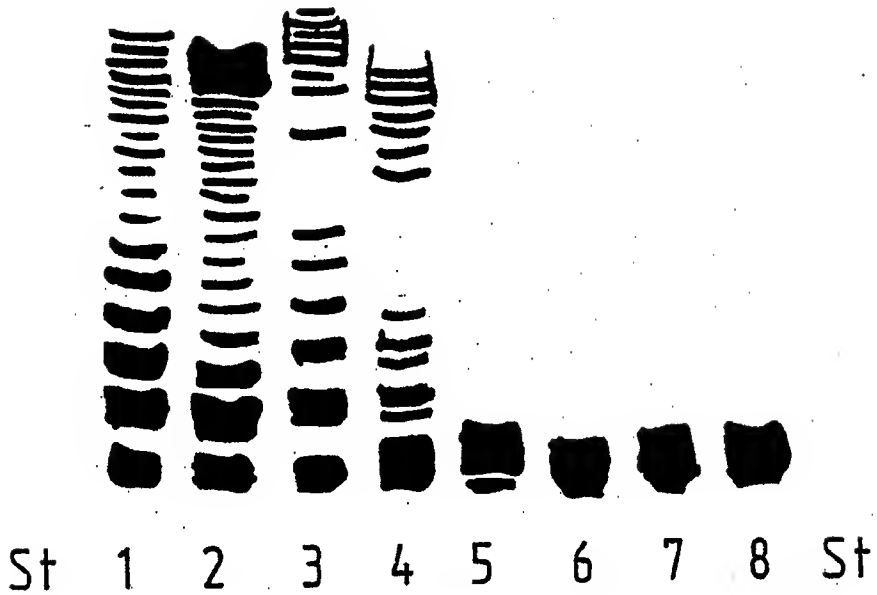
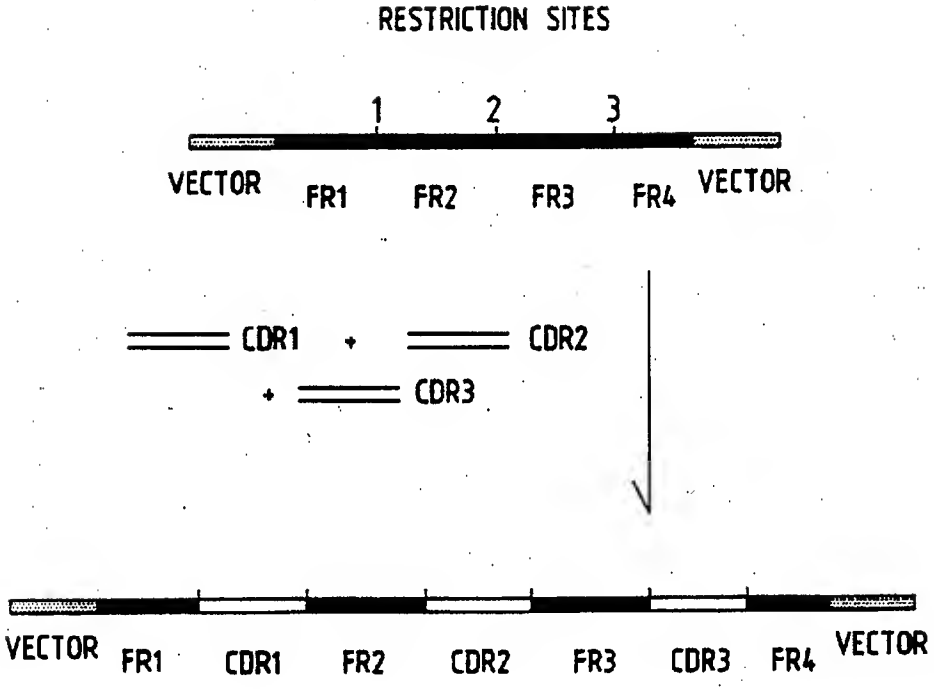


FIG. 5


RESTRICTION SITES
1
2
3
VECTOR
FR1
FR2
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FR4
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CDR1
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CDR2
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CDR3
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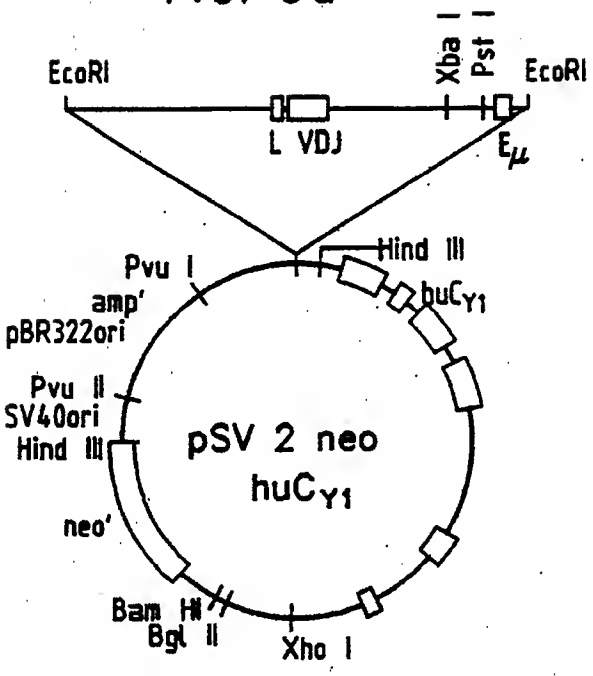
FR1
CDR1
FR2
CDR2
FR3
CDR3
FR4
VECTOR

FIG. 6a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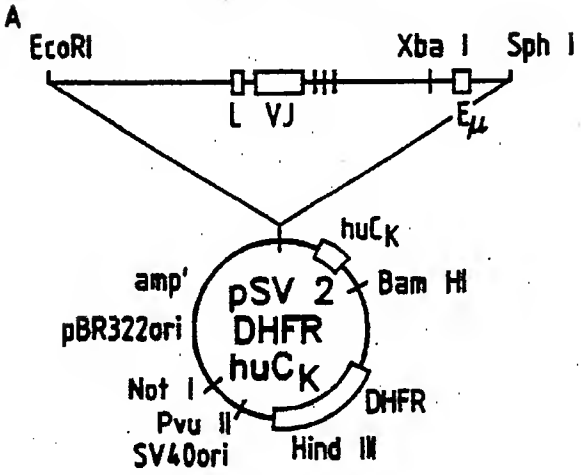
Xba I
Pst I
EcoRI
L VDJ
Eμ
Hind III
Pvu I
amp'
pBR322ori
huCγ1
Pvu II
SV40ori
Hind III
pSV 2 neo
huCγ1
neo'
Bam HI
Bgl II
Xho I

FIG. 6b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Xba I
Sph I
L VJ
Eμ
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huCK
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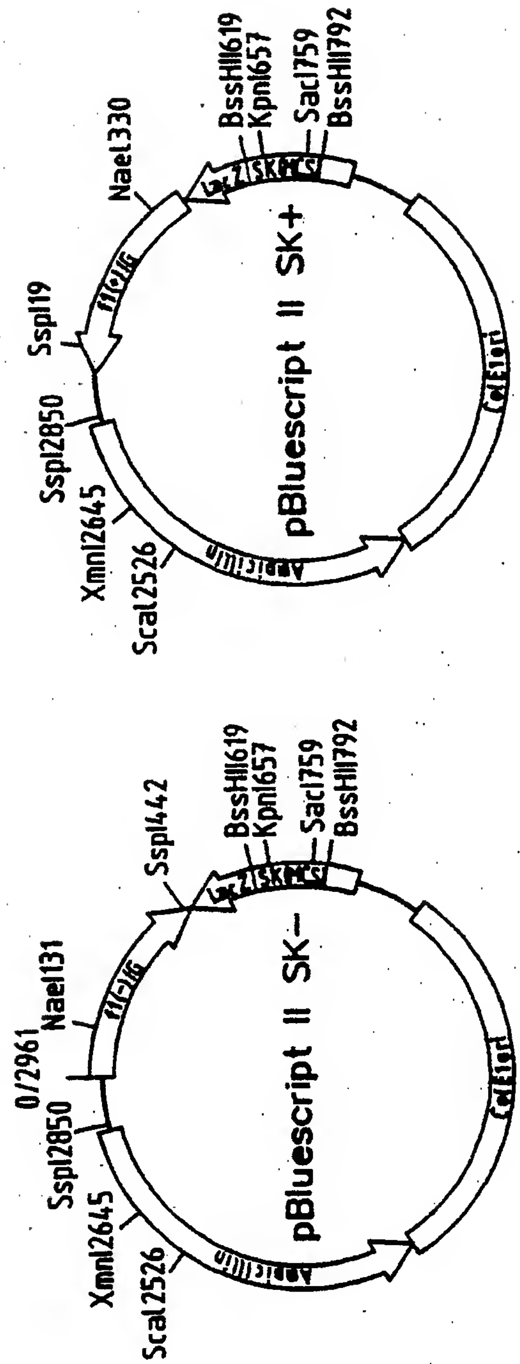
Hind III

FIG. 7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SspI19
SspI2850
XmnI2645
ScaI2526
BssHII619
KpnI657
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BssHII792
Ampicillin
ColE1 ori
pBluescript II SK−
SspI442
NaeI131
0/2961
SspI2850
XmnI2645
ScaI2526
BssHII619
KpnI657
SacI759
BssHII792
Ampicillin
ColE1 ori

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 92/00380

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/13 C 12 P 21/08 C 12 N 5/20
C 12 N 15/06 C 07 K 13/00 C 07 K 15/00 C 12 N 5/10
G 01 N 33/577 G 01 N 33/569 A 61 K 39/40

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K C 12 P C 12 N G 01 N A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,9003186 (CETUS CORP.) 5 April 1990, see claims; tables	1-10
Y	---	11-35
Y	EP,A,0239400 (G. WINTER) 30 September 1987, see the whole document (cited in the application)	11-35

X	WO,A,8404458 (M. POLLACK et al.) 22 November 1984, see page 23, line 12 - page 24, line 2; claims	1-10
Y		11-17, 34,35
	--- -/-	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19-05-1992	30. 06. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Natalia Wyttenbach

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	Nature, vol. 339, no. 6223, 1 June 1989, (London, GB), V.K. CHAUDHARY et al.: "A recombinant immunotoxin consisting of two antibody variable domains fused to Pseudomonas exotoxin", pages 394-397, see the abstract	11-17, 34,35
X	EP,A,0271379 (ROUSSEL-UCLAF) 15 June 1988, see the whole document	1-10
Y		11-17, 34,35
Y	Nature, vol. 341, no. 6242, 12 October 1989, (London, GB), E.S. WARD et al.: "Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli", pages 544-546, see the abstract	11-17, 34,35
X	EP,A,0286099 (VELOS GROUP) 12 October 1988, see the whole document	1-10
X	EP,A,0341684 (SUMITOMO CHEMICAL CO. et al.) 15 November 1989, see page 4, line 13 - page 15, line 38; claims	1,2,5-10
X	EP,A,0183876 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 11 June 1986, see the whole document	1,2,4-6 ,9-10
X	Proceedings of the National Academy of Sciences of USA, vol. 82, no. 6, March 1985, (Washington, DC, US), N.N.H. TENG et al.: "Protection against Gram-negative bacteremia and endotoxemia with human monoclonal IgM antibodies", pages 1790-1794, see the abstract	1,2,5,6 ,9,10
X	Journal of Infectious Diseases, vol. 162, no. 5, November 1990, (Chicago, IL, US), M. NYS et al.: "Protective effects of polyclonal sera and of monoclonal antibodies active to Salmonella minnesota Re595 lipopolysaccharide during experimental endotoxemia", pages 1087-1095, see the abstract	1-7,10

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9200380

SA 56439

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 12/06/92
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9003186	05-04-90	EP-A- 0434685	03-07-91
EP-A- 0239400	30-09-87	GB-A,B 2188638	07-10-87
		JP-A- 62296890	24-12-87
WO-A- 8404458	22-11-84	AU-B- 585200	15-06-89
		AU-A- 2869884	04-12-84
		EP-A,B 0151128	14-08-85
		EP-A- 0286099	12-10-88
		JP-T- 60501242	08-08-85
		US-A- 5057598	15-10-91
EP-A- 0271379	15-06-88	FR-A- 2606421	13-05-88
		JP-A- 1137993	30-05-89
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		EP-A,B 0151128	14-08-85
		JP-T- 60501242	08-08-85
		WO-A- 8404458	22-11-84
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		FR-A- 2631239	17-11-89
		GB-A- 2218703	22-11-89
		JP-A- 2084197	26-03-90
EP-A- 0183876	11-06-86	JP-A- 61130300	18-06-86

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9424449.8 2 December 1994 (02.12.94) GB

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).

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(54) Title: HUMANIZED ANTIBODIES TO CD38

(57) Abstract

The present invention relates to a monoclonal antibody, preferably with specificity for CD38, having CDRs of foreign origin and a recipient framework region having a sequence of human or primate origin, wherein the original amino acid residues in position 29 and/or 78 of the sequence of the recipient framework region of the heavy chain is replaced by a replacement amino acid residue that is the same or similar to that in the corresponding position of the sequence of the corresponding framework region of the heavy chain of the antibody from which the CDRs are derived. Method of preparation of said antibody. Pharmaceutical composition containing said antibody. Use of said antibody for the treatment of cancer and autoimmune diseases.

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Humanized antibodies to CD38

The present invention relates to antibodies and in particular to humanised antibodies and their preparation.

Antibodies typically comprise two heavy chains linked together by disulphide bonds and two light chains. Each light chain is linked to a respective heavy chain by disulphide bonds. Each heavy chain has at one end a variable domain followed by a number of constant domains. Each light chain has a variable domain at one end and a constant domain at its other end. The light chain variable domain is aligned with the variable domain of the heavy chain. The light chain constant domain is aligned with the first constant domain of the heavy chain. The constant domains in the light and heavy chains are not involved directly in binding the antibody to antigen.

The variable domains of each pair of light and heavy chains form the antigen binding site. The variable domains on the light and heavy chains have the same general structure and each domain comprises a framework of four regions, whose sequences are relatively conserved, connected by three complementarity determining regions (CDRs: CDRL1, CDRL2, CDRL3, CDRH1, CDRH2 and CDRH3). The four framework regions largely adopt a beta-sheet conformation and the CDRs form loops connecting, and in some cases forming part of, the beta-sheet structure. The CDRs are held together in close proximity by the framework regions and, with the CDRs from the other domain, contribute to the formation of the antigen binding site. The four framework regions are therefore crucial in ensuring the correct positioning of the CDRs relative to each other and hence in antibody binding.

The importance of the interaction between the CDRs and the framework regions has become increasingly evident as more and more non-human antibodies have become humanised, such humanised antibodies comprising non-human CDRs within a human framework. Humanised antibodies, in contrast to non-human antibodies, say mouse or rat antibodies, elicit a negligible immune response when administered to a human.

The prior art discloses several ways of producing such humanised antibodies. Thus EP-A-0239400 describes splicing CDRs into a human framework. Briefly, the CDRs are derived from a non-human species such as a rat or mouse whilst the framework regions of the variable domains, and the constant domains, are derived from a human antibody. Specifically, a humanised anti-CD52 antibody is disclosed in EP-A-0328404.

EP-A-054951 describes another way of humanising an antibody by re-shaping a non-human antibody to make it more like a human antibody. Basically, it comprises taking a non-human variable domain, such as mouse or rat variable domain, and changing the residues in the framework region to correspond to residues of a human framework.

In both EP-A-0239400 and EP-A-054951 an altered antibody is produced in which the CDRs of the variable domain of the antibody are derived from a first non-human species and the framework regions and, if present, the or each constant domain of the antibody are derived from human.

In such humanised antibodies a number of residues of the human framework region appear to exert a critical influence on the affinity of antigen binding (for example

Kettleborough *et al*, 1991, Prot. Eng. 4:773). Certain positions in the heavy chain framework regions, in particular, seem to be important in the retention of antigen-binding activity in a variety of altered antibodies. A number of investigators have reported the importance of residues at positions 67, 69 and 71, within the heavy chain framework region. These residues form a beta-sheet in contact with the interior aspect of the CDRH2 loop: presumably mismatches at these positions distort the CDR shape. Also, residues at positions 91 and 94 appear to be important for correct CDRH3 conformation in many heavy chains (for example Tempest *et al*, Bio/Technology 9:266). Other positions likely to affect antigen-binding are residues 27, 30 and 94 in the heavy chain, and residue 49 and 71 in the light chain (numbering according to the Kabat system). Furthermore, in the heavy chain the importance of regions 66-73 and 27-30 has been recognised in the literature, with residues 66-73 lying in close contact with CDRH2. It has now been found that the residues 29 and 78 of the framework region occupy a pocket which lies close to CDRH1 and affects antigen binding and that this undesirable effect can be obviated by using residues corresponding to those in the corresponding position of the framework region of the antibody from which the CDRs are derived.

Accordingly, the present invention is directed to a monoclonal antibody having donor CDRs of foreign origin and a recipient framework region having a sequence of human or primate origin, wherein the original amino acid residue in position 29 or 78 of the sequence of the recipient framework region of the heavy chain is replaced by a replacement amino acid that is the same or similar to that in the corresponding position of the sequence of

the corresponding framework region of the antibody from which the CDRs are derived. By "similar" is meant an amino acid of equivalent size preferably of equivalent size, hydrophobicity and charge.

Typically, the original amino acid residues in positions 29 and/or 78 of the recipient framework region are larger than their corresponding residues in the framework region of the antibody from which the CDRs are derived. Examples of these larger residues include tyrosine, histidine, tryptophan and 2-phenylalanine. Examples of the smaller corresponding residues in the framework region of the antibody donating the CDRs include glycine, alanine, valine, serine and leucine. In accordance with the invention, the larger original residue in positions 29 and/or 78 of the recipient framework is replaced with a replacement amino acid residue that is either the same or similar to the corresponding smaller residue of the antibody which is donating the CDRs.

Although it is preferable for the replacement amino acid residue to be the same as the corresponding residue of the antibody which is donating the CDRs it can also be a similar amino acid residue provided the character with respect to size and preferably also hydrophobicity and charge is essentially the same i.e. conserved. For example, if the residue of the antibody which is donating the CDRs has a valine in position 29 and/or 78, then instead of having a replacement amino acid residue in the recipient framework which is also valine, one could, for example, use alanine instead since alanine is of equivalent charge, size and hydrophobicity to valine and thus similar. The use of a similar amino acid in place of the exact same amino acid is a practice which is well established in the art and known as conservative

substitution.

By way of example, in a mouse heavy chain framework, side chains of Leu-29 and Val-78 would pack together in a small pocket close to CDRH1 whilst in the corresponding human heavy chain framework, such as for example NEW, which otherwise bears close homology to the mouse framework, the analogous positions are occupied by two Phe residues. The large aromatic side-chains appear to be too bulky to pack in the same fashion as in the mouse antibody and so alter the disposition of neighbouring surface residues resulting in a different conformation of CDRH1 in a humanised antibody. Substituting either Phe residue by the smaller murine residue partially relieves this effect allowing antigen binding. Full affinity is generally restored by replacement of both residues. It is therefore preferred that amino acids in both positions 29 and 78 are replaced.

In accordance with the invention, the replacement amino acid residues fit into the pocket without causing distortion of, for example, the CDRH1 conformation.

Preferably, the framework of the antibody heavy chain is homologous to the corresponding framework of the human antibody NEW (Saul *et al*, J. Biol. Chem. 253:585-597, 1978). The final residue of framework 1 in this case is suitably Ser or Thr, preferably Ser. This residue is at position 30 (Kabat *et al*, 1987). Preferably the framework of the antibody light chain is homologous to the variable domain framework of the protein REI (Epp *et al*, Eur. J. Biochem., 45:513-524, 1974).

Particular examples of murine heavy chains in which residues 29 and 78 pack together in a small pocket close

to CDRH1 are those in Kabat groups IB and IIC.

By contrast, other examples of human heavy chains which have bulky residues in positions 29 and 78 in the framework region are LES-C, T52, Ab44, HIgI and NEW, as listed in Kabat.

Species other than the mouse that may have residues of a small size in positions 29 and 78 are for example, the rat, rabbit and hamster.

All amino acid residue positions referred to herein employ the Kabat numbering system.

An antibody according to the invention may be produced by a method including the steps of:

(i) obtaining the sequence of a donor heavy chain;

(ii) selecting a recipient human or primate framework by best-fit homology method;

(iii) replacing the amino acid residue in position 29 or 78 of the sequence of the recipient framework region of the heavy chain by an amino acid that is the same or similar to that in the corresponding position of the sequence of the corresponding framework region of the antibody from which the CDRs are derived.

The antibody heavy chain may be co-expressed with a complementary antibody light chain. At least the framework regions of the variable domain and the or each constant domain of the complementary chain generally are derived from the primate or human recipient. Preferably

the CDRs of both chains are derived from the same selected antibody.

The antibody preferably has the structure of a natural antibody or a fragment thereof. The term antibody may therefore comprise a complete antibody, a $(Fab')_2$ fragment, a Fab fragment, Fv fragment, Fd fragment, SFv, a light chain dimer or a heavy chain and derivatives thereof. The antibody may be an IgG such as an IgG1, IgG2, IgG3 or IgG4, IgM, IgA, IgE or IgD. Furthermore, the antibody may comprise modifications of all classes e.g. IgG dimers, Fc mutants that no longer bind Fc receptors or mediate C1q binding (blocking antibodies). The antibody may also be a chimeric antibody of the type described in WO 86/01533) which comprises an antigen binding region and a non-immunoglobulin region. The antigen binding region is an antibody light chain variable domain or heavy chain variable domain. Typically, the antigen binding region comprises both light and heavy chain variable domains. The non-immunoglobulin region is fused at its C-terminus to the antigen binding region. The non-immunoglobulin region is typically a non-immunoglobulin protein and may be an enzyme, a toxin or a protein having known binding specificity. The two regions of the chimeric antibody may be connected via a cleavable linker sequence.

The invention is preferably employed to humanise an antibody, for example, an antibody of rat, rabbit, hamster or mouse origin. The framework regions and constant domains of the humanised antibody are therefore of human or primate origin whilst the CDRs of the light and/or heavy chain of the antibody are for example, rat or mouse CDRs. The antibody may be a human or primate IgG such as IgG1, IgG2, IgG3, IgG4; IgM; IgA; IgE or IgD

in which the CDRs are of rat or mouse origin.

The antibody from which the donor CDRs are derived is typically an antibody of a selected specificity. In order to ensure that this specificity is retained, either the variable domain framework regions of the antibody are re-shaped to correspond to variable domain framework regions of a human or primate antibody or the CDRs are grafted onto the closest human or primate framework regions. Either way, the resulting antibody preferably comprises non-human CDRs and human or primate framework regions that are homologous with the corresponding framework regions of the antibody from which the CDRs are derived. Preferably there is a homology of at least 50% between the two variable domains.

There are four general steps to produce a humanised antibody. These are:

(1) determining the nucleotide and predicted amino acid sequence of the light and heavy chain variable domains of the antibody from which the CDRs are derived;

(2) deciding which human or primate antibody framework region to use;

(3) the actual grafting or re-shaping methodologies/ techniques; and

(4) the transfection and expression of the grafted or re-shaped antibody.

These four steps are explained below.

Step 1: Determining the nucleotide and predicted amino acid sequence of the antibody light and heavy chain variable domains

To humanise an antibody the amino acid sequence of the non-human antibody's (donor antibody's) heavy and light chain variable domains needs to be known. The sequence of the constant domains is irrelevant. The simplest method of determining an antibody's variable domain amino acid sequence is from cloned cDNA encoding the heavy and light chain variable domain.

There are two general methods for cloning a given antibody's heavy and light chain variable domain cDNAs: (1) via a conventional cDNA library, or (2) via the polymerase chain reaction (PCR). Both of these methods are widely known. Given the nucleotide sequence of the cDNAs, it is a simple matter to translate this information into the predicted amino acid sequence of the antibody variable domains.

Step 2: Designing the humanised antibody

There are several factors to consider in deciding which human antibody (recipient antibody) sequence to use during humanisation. The humanisation of light and heavy chains are considered independently of one another, but the reasoning is basically the same.

This selection process is based on the following rationale: A given antibody's antigen specificity and affinity is primarily determined by the amino acid sequence of the variable region CDRs. Variable domain framework residues have little or no direct contribution. The primary function of the framework regions is to hold

the CDRs in their proper spacial orientation to recognise the antigen. Thus the substitution of rodent CDRs into a human variable domain framework is most likely to result in retention of the correct spacial orientation if the human variable domain is highly homologous to the rodent variable domain from which the CDRs were derived. A human variable domain should preferably be chosen therefore that is highly homologous to the rodent variable domain(s).

A suitable human antibody variable domain sequence can be selected as follows:

(i) Using a computer program, search all available protein (and DNA) databases for those human antibody variable domain sequences that are most homologous, for example, to the rodent antibody variable domains. This can be easily accomplished with a program called FASTA but other suitable programs are available. The output of the program is a list of sequences most homologous to the rodent antibody, the percent homology to each sequence, and an alignment of each sequence to the rodent sequence. This is done independently for both the heavy and light chain variable domain sequences. The above analyses are more easily accomplished if customised sub-databases are first created that only include human immunoglobulin sequences. This has two benefits. First, the actual computational time is greatly reduced because analyses are restricted to only those sequences of interest rather than all the sequences in the databases. The second benefit is that, by restricting analyses to only human immunoglobulin

sequences, the output will not be cluttered by the presence of rodent immunoglobulin sequences. There are far more rodent immunoglobulin sequences in databases than there are human.

(ii) List the human antibody variable domain sequences that have the most overall homology to the rodent antibody variable domain (from above). Do not make a distinction between homology within the framework regions and CDRs. Consider the overall homology.

(iii) Eliminate from consideration those human sequences that have CDRs that have a different length than those of the rodent CDRs. This rule does not apply to CDR 3, because the length of this CDR is normally quite variable. Also, there are sometimes no or very few human sequences that have the same CDR lengths as that of the rodent antibody. If this is the case, this rule can be loosened, and human sequences with one or more differences in CDR length can be allowed.

(iv) From the remaining human variable domains, one is selected that is most homologous to that of the rodent.

(v) The actual humanised antibody (the end result) should contain CDRs derived from the rodent antibody and a variable domain framework from the human antibody chosen above.

(vi) Instead of re-shaping or grafting to produce a humanised antibody, it would also be possible to synthesise the entire variable domain from

scratch once the amino-acids of the non-human variable domain has been determined and the most homologous human variable domain has been identified.

(vii) If donor heavy chain has two small residues at positions 29 and 78, and recipient chain has large, typically aromatic, residues at one or both of these positions, then further analysis is required.

(viii) This analysis may take the form of a sequence comparison between the CDRH1 of the donor chain and that of other antibodies. For example, a CDRH1 sequence of SYGVH has been shown to require small residues at positions 29 and 78 for complete activity, and it is to be expected that other antibodies with the same or similar CDRH1 sequence will also require residues at these positions.

Alternatively, the analysis may take the form of detailed computer aided modelling of the CDRH1 region of the proposed humanised antibody using standard techniques (for example the *AbM* package from Oxford Molecular Ltd). If this analysis, for example, reveals that CDRH1 lies in close approximation to the packed side chains of residues 29 and 78, and that altering these residues from human to smaller residues changes the orientation or position of CDRH1, then such smaller residues should replace the human ones. An example of such a perturbation of CDRH1 is shown in Figures 5 and 6.

Step 3: Grafting and re-shaping

See EP-A-0239400 and EP-A-054951 for details.

Step 4: The transfection and expression of the altered antibody

Once the antibody has been humanised and residues 29 and/or 78 replaced, the cDNAs are linked to the appropriate DNA encoding light or heavy chain constant region, cloned into an expression vector, and transfected into mammalian cells. These steps can be carried out in routine fashion. A humanised antibody may therefore be prepared by a process comprising:

(a) preparing a first replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least a variable domain of an Ig heavy or light chain, the variable domain comprising framework regions from a human or primate antibody and CDRs comprising at least parts of the CDRs from a second antibody of different origin;

(b) if necessary, preparing a second replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable domain of a complementary Ig light or heavy chain respectively;

(c) transforming a cell line with the first or both vectors; and

(d) culturing said transformed cell line to produce said altered antibody.

Preferably the DNA sequence in step (a) encodes both the variable domain and the or each constant domain of the antibody chain, the or each constant domain being derived from the human or primate antibody. The antibody can be recovered and purified. The cell line which is transformed to produce the altered antibody may be a Chinese Hamster Ovary (CHO) cell line or an immortalised mammalian cell line, which is advantageously of lymphoid origin, such as a myeloma, hybridoma, trioma, or quadroma cell line. The cell line may also comprise a normal lymphoid cell, such as a B-cell, which has been immortalised by transformation with a virus, such as the Epstein-Barr virus. Most preferably, the immortalised cell line is a myeloma cell line or a derivative thereof.

Although the cell line used to produce the altered antibody is preferably a mammalian cell line, any other suitable cell line, such as a bacterial cell line or a yeast cell line, may alternatively be used. In particular it is envisaged that E. coli-derived bacterial strains could be used.

Some immortalised lymphoid cell lines, such as myeloma cell lines, in their normal state secrete isolated Ig light or heavy chains. If such a cell line is transformed with the vector prepared in step (a), it may not be necessary to carry out step (b) of the process, provided that the normally secreted chain is complementary to the variable domain of the Ig chain encoded by the vector prepared in step (a). However, where the immortalised cell line does not secrete a complementary chain, it will be necessary to carry out (b). This step may be carried out by further manipulating the vector produced in step (a) so that this vector encodes not only the variable domain of an altered

antibody light or heavy chain, but also the complementary variable domain.

Alternatively, step (b) is carried out by preparing a second vector which is used to transform the immortalised cell line. This alternative leads to easier construct preparation, but may be less preferred than the first alternative in that it may not lead to as efficient production of antibody.

Where the immortalised cell line secretes a complementary light or heavy chain, the transformed cell line may be produced for example by transforming a suitable bacterial cell with the vector and then fusing the bacterial cell with the immortalised cell line by spheroplast fusion. Alternatively, the DNA may be directly introduced into the immortalised cell line by electroporation or other suitable method.

The present process has been applied to obtain an antibody against the CD38 surface antigen.

Briefly, a humanised anti-CD38 monoclonal antibody (termed h3S) was produced in the following fashion. cDNA was obtained from hybridoma cells secreting the murine monoclonal anti-(human CD38) AT13/5. cDNA clones encoding the heavy and light chains of the mouse antibody were identified and sequenced (Sequences 1 and 2 attached in Figures 1 and 2). This information was then used to choose appropriate human frameworks to receive the CDR grafts by the best-fit homology method. This procedure identified the REI light chain and the NEW heavy chain as the optimal choices.

CDRs were grafted on to the human frameworks. In

addition, guided by published work (Riechman et al., 1988 Nature 332: 323 and Tempest et al., 1991, Bio/Technology 9:266), four framework changes were made at this stage at positions likely to affect antigen-binding: residues 27,30 and 94 in the heavy chain, and residue 49 in the light chain (numbering according to the Kabat system). The resulting humanised antibody was tested for CD38 binding, with negative results. Expression of the humanised light chain together with a chimeric heavy chain (murine VH, human CH) produced functional antibody, indicating that the humanisation of the light chain was adequate.

A further series of heavy chain framework changes were examined. In particular, the analysis identified a stretch of sequence from residue 66 to 73 which lies in close contact with CDRH2 and a pocket formed by the side chains of residues 29 and 78, lying close to CDRH1, as affecting antigen binding. As mentioned earlier on the importance of the regions 66-73 and 27-30 is recognised in the literature, though the role of residue 29 and 78 and the interaction between the side chains of residues 29 and 78 is not.

Although the invention is described with reference to an anti-CD38 antibody it is applicable to any antibody, whatever antigen it binds to. In particular any antibodies that bind the 40kD antigen (CO/17.1.A) as disclosed in J. Cell. Biol., 125 (2) 437-446, April 1994 and in Proc. Natl. Acad. Sci. 87, 3542-3546, May 1990, carcinoma antigens and antigens involved in autoimmune diseases. A specific example of an anti-40KD antibody is 323/A3.

Another example of an antibody is an anti-folate receptor

antibody as disclosed in A. Tomasetti *et al*, Federation of European Biochemical Societies Vol 317, 143-146, Feb 1993. A specific example of an anti-folate antibody is MOV18. Further examples of antibodies include anti-CEA, anti mucin, anti-20/200KD, anti-ganglioside, anti-digoxin, anti-CD4 and anti-CD23.

In particular the anti-CD38 antibody has the nucleotide sequences for the heavy chain and light chain variable region as shown in Figures 3, 3a and 4.

According to another aspect of the present invention there is provided the use of antibody according to the present invention in therapy. In particular there is provided the use of antibodies according to the invention for the treatment of cancer and their associated metastases and for treatment of autoimmune diseases, in particular for the treatment of multiple myeloma, lymphoma and rheumatoid arthritis.

The anti-CD38 antibody of the present invention can be used in the treatment of multiple myeloma.

CD38 is a transmembrane glycoprotein expressed by immature B lymphocytes, activated T and B lymphocytes, and plasma cells. Antibodies to CD38 capable of causing cell lysis may be useful in the immunotherapy of tumours bearing this antigen, principally multiple myeloma and 50% of non-Hodgin's lymphomas. Additionally, anti-CD38 antibodies may be useful in the treatment of autoimmune diseases such as rheumatoid arthritis and myaethenia gravis, as they have the potential to suppress both the humoral and cellular effector arms of the immune system.

A CD38 antibody according to the present invention has

been demonstrated to be lytic for cells expressing CD38 on their surface. The humanised antibody has been shown to bind CD38 and compete with the parental antibody in CD38 binding.

Multiple myeloma is a neoplasm characterised by an accumulation of a clone of plasma cells, frequently accompanied by the secretion of immunoglobulin chains. Bone marrow invasion by the tumour is associated with anaemia, hypogammaglobinaemia and granulocytopaenia with concomitant bacterial infections. An abnormal cytokine environment, principally raised IL6 levels, often results in increased osteoclasis leading to bone pain, fractures and hypercalcaemia. Renal failure is not uncommon in the context of high concentrations of myeloma immunoglobulin and hypercalcaemia.

A variety of therapeutic protocols have been tried over recent years with little impact on the overall prognosis for myeloma patients. Treatment with melphalan and prednisolone remains the standard therapy, as it was thirty years ago (Bergsagel, 1989). A response to chemotherapy is associated with the induction of remission with median duration of about two years, but in all cases this is followed by eventual relapse and death (Alexanian and Dimopoulos, 1994 New England J. of Medicine Vol. 330 : 484). More aggressive chemotherapy utilising multiple cytotoxic agents has yielded little additional benefit in terms of survival or duration of remission, though high-dose therapy followed by autologous bone marrow transplant remains an area of active development.

Several workers have proposed immunotherapeutic strategies to combat myeloma. Interleukin 6 has been

suggested to be a major growth factor for myeloma cells and may function in either an autocrine or paracrine fashion. Based on such results, interventions aimed at disrupting the IL6 signalling system have been designed. Two murine monoclonal that neutralise IL6 suppressed the proliferation of myeloma cells in a patient with leukaemic variant of the disease, though the tumour relapsed after 60 days.

Administration of anti-IL6 receptor monoclonal antibody to SCID mice engrafted with cells from a human myeloma cell line suppressed tumour growth, though only if the antibody was administered one day after injection of the myeloma cells. Antibody given after five days of growth had no significant effect. A CDR-grafted form of this antibody has also been prepared for possible human therapeutic use.

In a similar vein, myeloma cells bearing high levels of IL6 receptor have also been targeted by chimeric cytotoxinx consisting of IL6 variants linked to a modified form of Pseudomonas exotoxin. Cell killing is seen in vitro though the applicability of this technique in the clinic remains to be seen.

Our preference is for a more physiological approach, targeting myeloma cells for killing by the host immune system. The surface antigen CD38 is strongly expressed by more than 90% of multiple myeloma cells, and its suitability as a target for lytic immunotherapy has been discussed (Stevenson *et al*, 1991 Blood, Vol. 77, 5 : 1071-1079). The same report also demonstrated the competence of effector cells from myeloma patients for lysis of target cells coated with a chimeric anti-CD38.

The dosages of such antibodies will vary with the condition being treated and the recipient of the treatment, but will be in the range 1 to about 100 mg for an adult patient, preferably 1 - 10 mg, usually administered daily for a period between 1 and 30 days. A two part dosing regime may be preferable wherein 1 - 5 mg are administered for 5 - 10 days followed by 6 - 15 mg for a further 5 - 10 days.

Also included within the invention are formulation containing a purified preparation of an anti-CD38 antibody. Such formulation preferably include, in addition to antibody, a physiologically acceptable diluent or carrier possibly in admixture with other agents such as other antibodies or antibiotic. Suitable carriers include but are not limited to physiological saline, phosphate buffered saline, phosphate buffered saline glucose and buffered saline. Alternatively, the antibody may be lyophilised (freeze-dried) and reconstituted for use when needed, by the addition of an aqueous buffered solution as described above. Routes of administration are routinely parenteral including intravenous, intramuscular, subcutaneous and intraperitoneal injection or delivery.

The following Examples illustrate the invention. In the accompanying drawings:

Figure 1 shows the nucleotide and predicted amino acid sequence of mouse anti-CD38 antibody heavy chain variable region. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. CDRs are underlined.

Figure 2 shows the nucleotide and predicted amino acid

sequence of mouse anti-CD38 antibody light chain variable region. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins respectively. CDRs (underlined) were identified by comparison to known immunological sequences (Kabat *et al*, "Sequences of proteins of immunologic interest", US Dept of Health and Human Services, US Government Printing Office, 1987).

Figures 3 and 3a together show the nucleotide and predicted amino acid sequence of the humanised anti-CD38 antibody light chain cDNA. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. CDRs are underlined.

Figure 4 shows the nucleotide and predicted amino acid sequence of the humanised anti-CD38 antibody heavy chain cDNA. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. CDRs are underlined.

Figure 5 shows the configuration of the CDRHI (dark tubes) in the murine-anti-CD38 (murine residues at positions 29 and 78).

Figure 6 shows the configuration of the CDRHI (dark tubes) in the same region as Figure 5, but in a humanised construct with human residues at positions 29 and 78.

Figure 7 shows the effect of various heavy chain framework substitutions on relative binding affinity of anti-CD38 antibodies.

Figure 8 shows the effect of various heavy chain

framework substitutions on antibody dependent cellular cytotoxicity mediated by CD38 antibodies.

Examples

Example 1

Humanisation of anti-CD38 based on a mouse antibody (AT13/5:IgGLK)

(a) General note on methodology

Unless otherwise stated, in the medodology described below, the following standard procedures and conditions were used. Manufacturers' recommended protocols were followed where applicable.

PCR experiments (Saiki *et al*, Science 239:487-491, 1988) were conducted using a programmable thermal cycler (Trio Biometra). A typical 100µl reaction mix contained 2.5 units of AmpliTaq polymerase (Perkin-Elmer Cetus, Beaconsfield, UK) in the buffer supplied by the manufacturer; 250µM of each of dATP, dCTP, DGTP and dTTP, amplification primers at 1 µM, and template DNA. Unless otherwise noted, the following cycle specifications were used:

step 0: 94°C for 90 seconds
step 1: 94°C for 60 seconds
step 2: 50°C for 60 seconds, ramping up to step 3 at a rate of 0.15°C/second
step 3: 72°C for 60 seconds, go to step 1, repeating this loop for 25 cycles
step 4: 72°C for 10 minutes.

DNA sequencing was performed by the dideoxy method using the Sequenase v2 system (USB, Cambridge, UK), according to the manufacturer's instructions. The reaction products were separated on 8% acrylamide sequencing gels (Gel-Mix 8, BRL, Paisley, Scotland, UK).

To gel-purify DNA, one of two methods was used. For fragments smaller than 175 base-pairs, the DNA was separated on a conventional high-melting point agarose gel, and the DNA recovered using the Prep-a-Gene system (Bio-Rad Laboratories, Hemel Hempstead, UK). Larger fragments were purified by separation on a low-melting point agarose gel (NuSieve GTG, FMC, Rockland, ME), and the DNA recovered using Magic PCR Preps (Promega, Southampton, UK).

Numbering of amino-acid residues in antibody chains follows the scheme of Kabat et al ("Sequences of proteins of immunological interest", US Dept of Health and Human Services, US Government Printing Office, 1991).

(b) Cloning and Sequencing of AT 13/5 antibody - Heavy Chain

Polyadenylated RNA was extracted from a culture containing 5×10^6 of the AT13/5 mouse hybridoma line using a Micro Fast Tract kit (British Biotechnology, Oxford, UK). This was converted into oligo-dT-primed single-stranded cDNA using the SuperScript Preamplification system (BRL, Paisley, Scotland, UK). Aliquots of the resulting cDNA were used in PCRs designed to separately amplify the variable region of mouse immunoglobulin heavy and light chains.

The variable region of the heavy chain was amplified

according to the method of Jones & Bendig (Bio/Technology 9:88-89), using a cocktail of primers specific to the signal peptide region (MHV1-12) and one primer specific for the mouse γ1 constant region (Mouse IgG1 heavy chain reverse primer). The resulting PCR fragment was digested with Xma I and Sal I and cloned into pUC18. Clones obtained from two independent PCR reactions were sequenced on both strands and found to be identical implying that the sequence does not contain errors introduced by the PCR process. The complete sequence of the variable region appears as Figure 1.

(c) Cloning and Sequencing of AT13/5 antibody - Light Chain

The sequence of the variable region of the light chain was also derived by a PCR-based cloning strategy using the same preparation of single-stranded cDNA as for the heavy chain. However, a more complex cloning and sequencing protocol was required, as the primers described by Jones & Bendig (*op cit*) preferentially amplify a non-productively rearranged kappa light chain from the AT13/5 cDNA. This chain arises from the fusion partner used to generate the AT13/5 hybridoma, here termed the MOPC-21 related V_K, and is of known sequence (Carroll, WL *et. al.*, Molecular Immunology 25:991-995; 1988).

To amplify the cDNA encoding the anti-CD38 light chain a PCR was performed using the mouse kappa light chain reverse primer described by Jones & Bendig (*op cit*), and a primer VK1-BACK that hybridises to the framework 1 region of most mouse kappa chains (sequences: 5' GACATTCAGCTGACCCAGTCTCCA 3'). Conditions were as described for the heavy chain amplifications above,

except that 35 cycles were used. These primers do not amplify the cDNA encoding the MOPC-21 related VK under these conditions.

An amplification fragment of the appropriate size was purified and a portion of this DNA used as the template for a second amplification (conditions as above, 30 cycles) using the light chain reverse primer and a variant of VK1-BACK containing a Hind III site (sequence: 5' GATCAAGCTTGACATTCAGCTGACCCAGTCTCCA 3'). The resulting fragment was digested with Hind III and Xma I and cloned into a pUC18. Clones were sequenced on both strands by the conventional dideoxy method. Additionally, a portion of the PCR product was directly sequenced using a thermal cycling strategy (fmol system, Promgea, Southampton, UK) with a primer (light chain reverse primer, as above) end-labelled with ^{32}P. The sequence obtained from the cycle sequencing experiment matched exactly the sequence derived by conventional methods.

Since this sequence was obtained from the products of two rounds of amplification, further confirmation of its accuracy was sought. The existing light chain sequence was used to design a primer that hydridises to the framework 1 region (sequence: 5' ACTAGTCGACCATCCTCCTTTTCTGTTTCTCTAGGAG 3'). This was used in conjunction with the light chain reverse primer in a PCR with the following cycle definition:

step 0: 95°C for 120 seconds
step 1: 95°C for 60 seconds
step 2: 50°C for 60 seconds
step 3: 72°C for 60 seconds, go to step 1, repeating this loop for 30 cycles
step 4: 72°C for 10 minutes

Three independent reactions were performed, and after purification, the products were digested by Xma I and Sal I, and cloned into pUC18. Several clones were sequenced by the dideoxy method. All sequences so obtained were identical to those obtained previously, confirming that the proposed light chain sequence was indeed free from PCR errors. The complete sequence of the variable region of the light chain appears as Figure 2.

(d) Design and construction of version 1 of the humanised antibody

Human variable domain frameworks were selected by the best-fit homology method (Lewis, AP & Crowe, JS in "Generation of Antibodies by Cell and Gene Immortalisation", Terhorst, C, Malavasi, F, Albertini, A (eds) Karger: Basel, 1993). The frameworks chosen for humanisation process were the light and heavy chain variable domains of Campath 1H (disclosed in EP-A-0328404). The humanised heavy and light chains were then constructed by a recombinant PCR technique (Lewis & Crowe, Gene 101:297-302, 1991).

i) Light Chain

The primers used in the humanisation process were:

A_L:5'GATCAAGCTTCTCTACAGTTACTGAGCACA3'
B_L:5'CCGATTATATATGTCCTCACTTGCCTTACAGGTGATGGTCAC3'
C_L:5'AGTGAGGACATATATAATCGGTTAACCTGGTACCAGCAGAAG3'
D_L:5'AGTTTCCAAACTGGTTGCACCAGAGATCAGCAGCTTTGG3'
E_L:5'GGTGCAACCAGTTTGGAAACTGGTGTGCCAAGCAGA3'
F_L:5'GTACGGATTACTCCAATACTGTTGGCAGTAGTAGGTGGC3'
G_L:5'CAGTATTGGAGTAATCCGTACACGTTCGGCCAAGGGACC3'
H_L:5'GATCAAGCTTCTAACACTCTCCCCTGTTGA3'

Primers A_L and H_L contain Hind III sites to allow cloning of the final amplficiation product. PCRs were performed according to the following cycle specification:

step 0: 95°C for 120 seconds
step 1: 95°C for 60 seconds
step 2: 45°C for 60 seconds
step 3: 72°C for 60 seconds, go to step 1, repeating this loop for 25 cycles
step 4: 72°C for 10 minutes

The template used in this reaction was DNA encoding the Campath 1H light chain, a construct in which the framework residues are taken from REI and the CDRs from a rat anti-human CDw52 antibody (Reichmann, L. *et. al.* Nature 332:323-337, 1988). The primers above are designed to wholly replace the Campath 1H sequence, leaving the AT13/5 CDRs grafted onto the REI frameworks.

Four initial PCRs were performed using 10ng of template with the primer pairs: A_L and B_L, C_L and D_L, E_L and F_L, and G_L and H_L. The products of these reactions, AB_L, CD_L, EF_L and GH_L respectively were gel-purified and half of the amount recovered used in the second round of PCRs. Fragments AB_L and CD_L were used as template with primers A_L and D_L in one reaction, and fragments EF_L and GH_L were used as template with primers E_L and H_L. The reacton conditions were:

step 0: 95°C for 120 seconds
step 1: 95°C for 60 seconds
step 2: 45°C for 60 seconds
step 3: 72°C for 90 seconds, go to step 1, repeating this loop for 20 cycles

The products of these reactions, AD_L and EH_L, were gel-purified and half of each DNA used as template in a final reaction with primers A_L and H_L with the reaction conditions as for the second round PCR above. The resulting product was digested with Hind III and cloned into pUC18. A clone with the predicted structure as determined by complete sequence of the insert on both strands was chosen for further manipulation. The sequence of the variable region of this construct is given as Figures 3 and 3a.

ii) Heavy Chain

The primers used in the humanisation process were:

A_H:5'GATCAAGCTTTACAGTTACTCAGCACACAG3'

B_H:5'GTGGACACCATAACTGGTGAAGGTGAAGCC3'

C_H:5'AGTTATGGTGTCCACTGGGTGAGACAGCCA3'

D_H:5'TTGTAGTCTGTGCTTCCACCTCTCCACATCACTCCAATCCACTCAAG3'

E_H:5'GAAGCACAGACTACAATGCAGCTTTCATGTCCAGAGTGACAATGCTG3'

F_H:5'GGAGTCCATCACGAAGCCGGTCGTTATCATGGATTTTGCACAATAATAGA C3'

G_H:5'AAATCCATGATAACGACCGGCTTCGTGATGGACTCCTGGGGTCAAGGCTC ACTAGTCACAGTCTCCTCAGCC3'

H_H:5'TAGAGTCCTGAGGGAATTCGGACAGCCGGGAAGGTG3'

PCRs were performed according to the following cycle specification:

step 0: 95°C for 120 seconds
step 1: 95°C for 60 seconds
step 2: 45°C for 60 seconds
step 3: 72°C for 60 seconds, go to step 1, repeating this loop for 25 cycles
step 4: 72°C for 10 minutes

The template used in this reaction was DNA encoding the Campath 1H heavy chain, a construct in which the CDRs and framework residues 27 and 30 are taken from a rat anti-human CDw52 antibody (Reichmann, L *et. al.* op cit), and the remainder of the framework residues from NEW. The primers above are designed to replace the Campath 1H CDR sequences, leaving the AT13/5 CDRs grafted onto the Campath 1H framework. Also, heavy chain residue 94 is known to be important in antigen-binding (Tempest, PR *et. al.*, Bio/Technology, 9:260-271, 1991), so the AT13/5 sequence was adopted at this position. The rat sequence at residues 27 and 30 is more homologous to the AT13/5 sequence than is the unmodified NEW sequence. Primers A_H and H_H contains Hind III and EcoR I sites respectively. Additionally, primer G_H engineers a SpeI site into the framework 4 region to allow coupling to a previously prepared human C_H sequence.

Four initial PCRs were performed using 10ng of template with the primer pairs: A_H and B_H, C_H and D_H, E_H and F_H, and G_H and H_H. The products of these reactions, AB_H, CD_H were used as template with primers A_H and D_H in one reaction, and fragments EF_H and GH_H were used as template with primers E_H and H_H. The reaction conditions were:

step 0: 95°C for 120 seconds
step 1: 95°C for 60 seconds
step 2: 45°C for 60 seconds
step 3: 72°C for 90 seconds, go to step 1, repeating this loop for 20 cycles

The products of these reactions, AD_H and EH_H, were gel-purified and half of each DNA used as template in a final reaction with primers A_H and H_H with the reaction conditions as for the second round PCR above. The

resulting product was digested with Hind III and Spe I, and the fragment containing the variable region cloned into a pUC18-based vector containing the human C_H sequence. A clone with the predicted structure as determined by complete sequencing of the insert on both strands was chosen for further manipulation.

(e) Eukaryotic expression of version 1 of the humanised antibody

Humanised AT13/5 heavy and light chains were cloned into eukaryotic expression vectors under human β actin promoters. The heavy and light chain plasmids were transiently expressed in B11 CHO cells by cotransfection of the two plasmids using Transfectam (Promega, Southampton, UK). Culture supernatants were assayed for human IgG by ELISA, and tested for CD38-binding activity by FACS analysis using the CD38-positive B-cell line Wien 133.

Although the culture supernatants contained significant amounts of human IgG, no anti-CD38 activity could be detected by FACS, even when supernatants were concentrated 10-fold. This result suggests that simple grafting of the CDRs from AT13/5 onto the Campath 1H and REI human frameworks is insufficient to transfer the antibody specificity. A series of framework changes were therefore undertaken in order to restore CD38-binding activity.

(f) Framework changes

Since most of the framework residues previously shown to be important in restoring antigen binding are in the heavy chain variable region, it was decided to focus on

this part of the antibody. Additional cotransfection of the humanised light chain with a chimaeric heavy chain construct (mouse heavy variable region fused to human C_H), produced active antibody (hereafter termed hybrid antibody) that bound CD38 with an affinity comparable to that of the original mouse antibody. The region with the lowest homology between the human frameworks used and the original mouse sequence is also close to some residues of known importance. This region, just downstream of the CDR3 sequence was chosen for mutagenesis.

Heavy chain residues 67 to 71 inclusive and 73 were grafted from the mouse antibody onto the humanised heavy chain using recombinant PCR. The primers used were as follows:

A_H: sequence as above
I_H: 5'GTTGTCCTTGGTGATGTTCAGTCTGGACATGAAAGCTGC3'
J_H: 5'CTGAACATCACCAAGGACAACAGCAAGAACCAGTTCAGC3'
H_H: sequence as above.

Two initial PCRs were performed using 10ng of version 1 humanised heavy chain template with the primer pairs: A_H and I_H and J_H and H_H. The products of these reactions, AI_H and JH_H respectively, were gel-purified and half of the recovered DNA used in a second round of PCR with primers A_H and H_H to generate version 2 of the humanised heavy chain variable region. This was cloned, sequenced, transferred to the expression system, and then transiently co-expressed with the humanised light chain construct as above. Once again, culture supernatant from transfected CHO cells produced human IgG as determined by ELISA, but no CD38-binding activity could be detected by FACS analysis.

A further round of mutations based on both version 1 and version 2 of the humanised heavy chain were then produced by a method identical to that described above. A total of six version 3 heavy chains were produced in which the following heavy chain framework residues were grafted from the mouse sequence onto one or other humanised sequence:

Antibody	Template for mutagenesis	Grafted residues	Primers used
h3J	version 1	28,29	K_H,L_H
h3K	version 2	28,29	K_H,L_H
h3L	version 1	76	M_H,O_H
h3M	version 2	76	N_H,O_H
h3N	version 1	28,29,76	K_H,L_H,M_H,O_H
h3O	version 2	28,29,76	K_H,L_H,N_H,O_H

Additionally, all constructions used primers A_H and H_H. The primer sequences used were:

A_H: sequence as above

H_H: sequence as above

K_H: 5'ACTGGTTAACGAAAAGCCAGACACGGTGCAGGTCAG3'

L_H: 5'GGCITTTCGTTAACCAGTTATGGTGTCCACTGGGTG3'

M_H: 5'AAATTGCCGTTTCGAAGTGTCTACCAGCATTGTCAC3'

N_H: 5'AAATTGCCGTTTCGAATTGTCCTTGGTGATGTTCAG3'

O_H: 5'TTCGAAACGGCAATTTAGCTTGAGACTCAGCAGC3'

Heavy chain constructs containing the expected sequence were transferred into mammalian expression vectors, and cotransfected with the humanised light chain construct into CHO cells, as above. Tissue culture supernatants containing human IgG as determined by ELISA were assayed for CD38-binding activity by FACS. Constructs h3K and h3O showed antigen-binding in this assay though with less

activity than the hybrid antibody (see Fig. 7).

(g) Method for changing framework residues at positions 29 and 78

In order to establish why h30 showed less activity than the hybrid antibody further sequences analysis suggested potential problems with positions 29 and 78 in the heavy chain.

Having identified mutations to be made in the heavy chain framework regions, these can be produced by a variety of standard methods: examples being site-directed mutagenesis, recombinant PCR and gene synthesis using oligonucleotides. In the case of the anti-CD38 heavy chain VH, recombinant PCR was used to introduce murine residues at positions 28-29 and 78 sequentially.

A human anti-CD38 heavy chain VH already incorporating murine residues at positions 27, 30, 67, 68, 69, 70, 71, 73 and 94 (Version 2 as described in (f) above) was used as template for the first round of mutagenesis. This was amplified with the following PCR primers in two separate reactions:

Primer A: 5'GATCAAGCTTTACAGTTACTCAGCACAG3'
Primer B: 5'ACTGGTTAACGAAAAGCCAGACACGGTGCAGGTCAG3'
Primer C: 5'GGCTTTTCGTTAACCAGTTATGGTGTCCACTGGGTG3'
Primer D: 5'TAGAGTCCTGAGGGAATTCGGACAGCCGGGAAGGTG3'

In primers B and C, the triplets encoding the murine residues at positions 28 and 29 are underlined. In the first reaction, the template was amplified with primers A and B. In the second reaction, the template was amplfified with primers C and D. The products of the two reactions were purified, mixed, and amplified with primers A and D. The reaction product was purified,

cleaved with Hind III and SpeI, and the 450 base-pair fragment encoding the VH cloned into a variant of pUC18 containing a human γ1 cDNA cassette (Sime *et al*, 1993; J. Immunol, 151:2296). Clones were sequenced to ensure correct introduction of the murine residues at positions 28 and 29.

A clone incorporating these changes was then used as template for a second round of recombinant PCR mutagenesis to introduce the murine residue at position 78. A procedure identical to that described above was followed, except that primers B and C were replaced by primers E and F respectively, which contain a triplet (underlined) that incorporates the murine residue at position 78.

Primer E: 5'AACCAGGTGAGCTTAAGACTCAGCAGCGTGACA3'
Primer F: 5'TCTTAAGCTCACCTGGTTCTTGCTGTTGTCCTT3'

The resulting heavy chain (see Fig. 4) when co-expressed with the humanised light chain (see Fig. 3) produces humanised anti-CD38,h3S.

(h) Eukaryotic expression of functional humanised antibody

To creat clonal cell lines for further characterisation, plasmids encoding the humanised h3S heavy chain and the chimaeric heavy chain were separately co-transfected with the humanised light chain into B11 CHO cells.

Example 2

Biological activity

(a) CD38 Binding Studies

(i) Effect of various heavy chain framework substitutions on relative binding affinity of anti-CD38 antibodies.

Binding was assessed by FACS staining of CD38 positive cells.

Heavy chains incorporating one or more of mouse framework residues were created as described above and combined with the humanised light chain to make antibodies which were assayed for binding to CD38, with the following results.

Construct	66-73	28/29	78	Binding
h1	-	-	-	-
h2	+	-	-	-
h3J	-	+	-	-
h3K	+	+	-	+
h3S	+	+	+	++

In this table, + denotes that the murine framework residue is incorporated into the humanised antibody at the indicated position, - denotes that the human residue remains.

Discussion

According to computer modelling studies the change of the 66-73 region back to mouse framework causes the humanised CDRH2 to adopt a similar conformation to that of the mouse antibody. However, as the construct h2 shows, this is insufficient to obtain binding. The model also suggests that in the mouse anti-CD38 antibody, positions 29 and 78 are occupied by small residues, whose side-

chains pack neatly together allowing CDRH1 to adopt the correct configuration for antigen binding. In the humanised constructs h1 and h2, the side chains are unable to pack together in this fashion, being much larger, and so distort CDRH1, preventing antigen binding. This aspect of the model is illustrated in Figures 5 and 6 (attached). Figure 5 shows the configuration of CDRH1 (dark tubes) in the murine anti-CD38. In Figure 6 showing the same region in a humanised construct with human residues at positions 29 and 78, the extra bulk of these side chains has clearly resulted in a distortion of the CDRH1 conformation.

Partial relief of this effect can be obtained by using the murine residue at position 29 and the human residue at position 78, though the resulting antibody shows markedly reduced function. Use of murine residues at both positions 29 and 78 restores activity, as evidenced by the data for the h3S construct.

(ii) Anti-CD38 heavy chain variable regions were fused to human γ1 constant region and coexpressed in CHO cells with humanised anti-CD38 light chain. CD38-binding activity is expressed normalised to the signal obtained using a saturating dose of hybrid antibody (mouse VH) in the same experiment.

Results are shown in Figure 7 where:

- ♦ Humanised antibody with murine residues at 28,29 and 78
- ▲ Humanised antibody with murine residues at 28,29 and 76

- Humanised antibody with murine residues at 28,29
- Hybrid antibody

In addition to the above substitutions, all humanised heavy chains contained murine framework residues at positions 27, 30, 67, 68, 69, 70, 71, 73 and 94. These alone are insufficient to obtain detectable binding by FACS.

These results demonstrate the critical importance of the small residues at positions 29 and/or 78 in obtaining full humanised heavy chain activity. They also demonstrate the specific nature of the interaction, in that a murine residue at position 76 close to position 78 was unable to restor activity.

(b) Effect of various heavy chain framework substitutions on antibody-dependent cellular cytotoxicity mediated by CD38 antibodies.

Antibody-dependent cellular cytotoxicity is normally assessed by one of several label-release techniques, well-known in the literature. In one such assay, 10^4 target cells (Wien 133) were labelled with europium and then exposed to freshly prepared human peripheral blood lymphocytes in the presence of antibody as an effector:target ratio of 50:1. Lysis was estimated by detecting release of europium after 4 hours, and quantitated by reference to control reactions without antibody or peripheral blood lymphocytes or with detergent such as Triton-X100.

The effect of framework substitutions on the lytic potential of humanised anti-CD38 monoclonals was examined

in label-release assay. Wien 133 target cells were loaded with label (either 51Cr or Eu) and then exposed to freshly prepared human peripheral blood mononuclear cells in the presence of varying amounts of anti-CD38 antibody. Cytotoxicity is expressed as the proportion of total releasable label liberated by antibody treatment.

Results are shown in Figure 10 where:

- ▲ Humanised antibody with murine residues at 28,29 and 78
- ■ Humanised antibody with murine residues at 28,29 and 76
- ● Hybrid antibody

These results show that the combination of framework changes at positions 29 and 78 confer full activity on the humanised heavy chain for cytotoxic function. Although incorporation of a small murine residue at position 29 results in some binding activity (Figure 7), this is insufficient to achieve full effector function.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: The Wellcome Foundation Limited
(B) STREET: Unicorn House, PO Box 129, 160 Euston Road
(C) CITY: London
(D) STATE: not applicable
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): NW1 2BP

(A) NAME: ELLIS, Jonathan Henry
(B) STREET: The Wellcome Research Laboratories, Langley Court
(C) CITY: South Eden Park Road,Beckenham
(D) STATE: Kent
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): BR3 3BS

(A) NAME: LEWIS, Alan Peter
(B) STREET: The Wellcome Research Laboratories, Langley Court
(C) CITY: South Eden Park Road,Beckenham
(D) STATE: Kent
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): BR3 3BS

(ii) TITLE OF INVENTION: ANTIBODIES

(iii) NUMBER OF SEQUENCES: 46

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 454 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..453

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
GGT CGA CTG GCT GTG TTA GCG CTG CTC TTC TGC CTG GTG ACA TTC CCA      48
Gly Arg Leu Ala Val Leu Ala Leu Leu Phe Cys Leu Val Thr Phe Pro
 1               5                  10                  15

AGC TGT GTC CTG TCC CAG GTG CAG CTG AAG CAG TCA GGA CCT GGC CTA      96
Ser Cys Val Leu Ser Gln Val Gln Leu Lys Gln Ser Gly Pro Gly Leu
            20                  25                  30

GTG CAC CCC TCA CAG AGC CTG TCC ATA ACC TGC ACA GTC TCT GGT TTC     144
Val His Pro Ser Gln Ser Leu Ser Ile Thr Cys Thr Val Ser Gly Phe
        35                  40                  45

TCA TTA ACT AGT TAT GGT GTC CAC TGG GTT CGC CAG TCT CCA GGA AAG     192
```

```
Ser Leu Thr Ser Tyr Gly Val His Trp Val Arg Gln Ser Pro Gly Lys
 50                  55                  60

GGT CTG GAG TGG CTG GGA GTG ATG TGG AGA GGT GGA AGC ACA GAC TAC      240
Gly Leu Glu Trp Leu Gly Val Met Trp Arg Gly Gly Ser Thr Asp Tyr
 65                  70                  75                  80

AAT GCA GCT TTC ATG TCC AGA CTG AAC ATC ACC AAG GAC AAC TCC AAG      288
Asn Ala Ala Phe Met Ser Arg Leu Asn Ile Thr Lys Asp Asn Ser Lys
                 85                  90                  95

CGC CAG GTT TTC TTT AAA ATG AAC AGT CTA CAA GCT GAT GAC ACT GCC      336
Arg Gln Val Phe Phe Lys Met Asn Ser Leu Gln Ala Asp Asp Thr Ala
            100                 105                 110

ATA TAC TAC TGT GCC AAA TCG ATG ATT ACG ACG GGC TTT GTT ATG GAC      384
Ile Tyr Tyr Cys Ala Lys Ser Met Ile Thr Thr Gly Phe Val Met Asp
        115                 120                 125

TCC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA GCC AAA ACG ACA      432
Ser Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser Ala Lys Thr Thr
    130                 135                 140

CCC CCA TCT GTC TAT CCA CTG G                                       454
Pro Pro Ser Val Tyr Pro Leu
145                 150
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 151 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gly Arg Leu Ala Val Leu Ala Leu Leu Phe Cys Leu Val Thr Phe Pro
1 5 10 15

Ser Cys Val Leu Ser Gln Val Gln Leu Lys Gln Ser Gly Pro Gly Leu
20 25 30

Val His Pro Ser Gln Ser Leu Ser Ile Thr Cys Thr Val Ser Gly Phe
35 40 45

Ser Leu Thr Ser Tyr Gly Val His Trp Val Arg Gln Ser Pro Gly Lys
50 55 60

Gly Leu Glu Trp Leu Gly Val Met Trp Arg Gly Gly Ser Thr Asp Tyr
65 70 75 80

Asn Ala Ala Phe Met Ser Arg Leu Asn Ile Thr Lys Asp Asn Ser Lys
85 90 95

Arg Gln Val Phe Phe Lys Met Asn Ser Leu Gln Ala Asp Asp Thr Ala
100 105 110

Ile Tyr Tyr Cys Ala Lys Ser Met Ile Thr Thr Gly Phe Val Met Asp
115 120 125

Ser Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser Ala Lys Thr Thr
130 135 140

Pro Pro Ser Val Tyr Pro Leu
145 150

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 454 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCAGCTGACC GACACAATCG CGACGAGAAG ACGGACCACT GTAAGGGTTC GACACAGGAC 60

AGGGTCCACG TCGACTTCGT CAGTCCTGGA CCGGATCACG TGGGGAGTGT CTCGGACAGG 120

TATTGGACGT GTCAGAGACC AAAGAGTAAT TGATCAATAC CACAGGTGAC CCAAGCGGTC 180

AGAGGTCCTT TCCCAGACCT CACCGACCCT CACTACACCT CTCCACCTTC GTGTCTGATG 240

TTACGTCGAA AGTACAGGTC TGACTTGTAG TGGTTCCTGT TGAGGTTCGC GGTCCAAAAG 300

AAATTTTACT TGTCAGATGT TCGACTACTG TGACGGTATA TGATGACACG GTTTAGCTAC 360

TAATGCTGCC CGAAACAATA CCTGAGGACC CCAGTTCCTT GGAGTCAGTG GCAGAGGAGT 420

CGGTTTTGCT GTGGGGGTAG ACAGATAGGT GACC 454

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 364 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..363

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
GAC ATT CAG CTG ACC CAG TCT CCA TCC TCC TTT TCT GTT TCT CTA GGA      48
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Phe Ser Val Ser Leu Gly
          155                 160                 165

GAC AGA GTC ACC ATT ACT TGC AAG GCA AGT GAG GAC ATA TAT AAT CGG      96
Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Glu Asp Ile Tyr Asn Arg
        170                 175                 180

TTA ACC TGG TAT CAG CAG AAA CCA GGA AAT GCT CCT AGG CTC TTA ATA     144
Leu Thr Trp Tyr Gln Gln Lys Pro Gly Asn Ala Pro Arg Leu Leu Ile
    185                 190                 195

TCT GGT GCA ACC AGT TTG GAA ACT GGG GTT CCT TCA AGA TTC AGT GGC     192
Ser Gly Ala Thr Ser Leu Glu Thr Gly Val Pro Ser Arg Phe Ser Gly
200                 205                 210                 215

AGT GGA TCT GGA AAG GAT TAC ACT CTC AGC ATT ACC AGT CTT CAG ACT     240
Ser Gly Ser Gly Lys Asp Tyr Thr Leu Ser Ile Thr Ser Leu Gln Thr
                220                 225                 230

GAA GAT GTT GCT ACC TAT TAC TGT CAA CAG TAT TGG AGT AAT CCG TAC     288
Glu Asp Val Ala Thr Tyr Tyr Cys Gln Gln Tyr Trp Ser Asn Pro Tyr
          235                 240                 245
```

```
ACG TTC GGA GGG GGG ACC AAG CTG GAA ATA AGA CGG GCT GAT GCT GCA      336
Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Arg Arg Ala Asp Ala Ala
        250                 255                 260

CCA ACT GTA TCC ATC TTC CCA CCA TCC A                               364
Pro Thr Val Ser Ile Phe Pro Pro Ser
    265                 270
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 121 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Phe Ser Val Ser Leu Gly
 1               5                  10                  15

Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Glu Asp Ile Tyr Asn Arg
            20                  25                  30

Leu Thr Trp Tyr Gln Gln Lys Pro Gly Asn Ala Pro Arg Leu Leu Ile
        35                  40                  45

Ser Gly Ala Thr Ser Leu Glu Thr Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Lys Asp Tyr Thr Leu Ser Ile Thr Ser Leu Gln Thr
65                  70                  75                  80

Glu Asp Val Ala Thr Tyr Tyr Cys Gln Gln Tyr Trp Ser Asn Pro Tyr
```

85 90 95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Arg Arg Ala Asp Ala Ala
100 105 110

Pro Thr Val Ser Ile Phe Pro Pro Ser
115 120

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 364 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CTGTAAGTCG ACTGGGTCAG AGGTAGGAGG AAAAGACAAA GAGATCCTCT GTCTCAGTGG 60

TAATGAACGT TCCGTTCACT CCTGTATATA TTAGCCAATT GGACCATAGT CGTCTTTGGT 120

CCTTTACGAG GATCCGAGAA TTATAGACCA CGTTGGTCAA ACCTTTGACC CCAAGGAAGT 180

TCTAAGTCAC CGTCACCTAG ACCTTTCCTA ATGTGAGAGT CGTAATGGTC AGAAGTCTGA 240

CTTCTACAAC GATGGATAAT GACAGTTGTC ATAACCTCAT TAGGCATGTG CAAGCCTCCC 300

CCCTGGTTCG ACCTTTATTC TGCCCGACTA CGACGTGGTT GACATAGGTA GAAGGGTGGT 360

AGGT 364

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 746 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:3..737

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AA GCT TCT CTA CAG TTA CTG AGC ACA CAG GAC CTC ACC ATG GGA TGG 47
Ala Ser Leu Gln Leu Leu Ser Thr Gln Asp Leu Thr Met Gly Trp
125 130 135

AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGT GTC CAC TCC 95
Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly Val His Ser
140 145 150

GAC ATC CAG ATG ACC CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT 143
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
155 160 165

GAC AGA GTG ACC ATC ACC TGT AAG GCA AGT GAG GAC ATA TAT AAT CGG 191
Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Glu Asp Ile Tyr Asn Arg
170 175 180

```
TTA ACC TGG TAC CAG CAG AAG CCA GGT AAG GCT CCA AAG CTG CTG ATC      239
Leu Thr Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
185                 190                 195                 200

TCT GGT GCA ACC AGT TTG GAA ACT GGT GTG CCA AGC AGA TTC AGC GGT      287
Ser Gly Ala Thr Ser Leu Glu Thr Gly Val Pro Ser Arg Phe Ser Gly
                205                 210                 215

AGC GGT AGC GGT ACC GAC TTC ACC TTC ACC ATC AGC AGC CTC CAG CCA      335
Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro
            220                 225                 230

GAG GAC ATC GCC ACC TAC TAC TGC CAA CAG TAT TGG AGT AAT CCG TAC      383
Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Trp Ser Asn Pro Tyr
        235                 240                 245

ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA CGA ACT GTG GCT GCA      431
Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
    250                 255                 260

CCA TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA      479
Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
265                 270                 275                 280

ACT GCC TCT GTT GTG TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG GCC      527
Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
                285                 290                 295

AAA GTA CAG TGG AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC TCC CAG      575
Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
            300                 305                 310

GAG AGT GTC ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC      623
Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
        315                 320                 325
```

```
AGC ACC CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC      671
Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
    330                 335                 340

GCC TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC      719
Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
345                 350                 355                 360

TTC AAC AGG GGA GAG TGT TAGAAGCTT                                    746
Phe Asn Arg Gly Glu Cys
                365
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 245 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
Ala Ser Leu Gln Leu Leu Ser Thr Gln Asp Leu Thr Met Gly Trp Ser
 1               5                  10                  15

Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly Val His Ser Asp
            20                  25                  30

Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp
        35                  40                  45

Arg Val Thr Ile Thr Cys Lys Ala Ser Glu Asp Ile Tyr Asn Arg Leu
    50                  55                  60
```

Thr Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Ser
65 70 75 80

Gly Ala Thr Ser Leu Glu Thr Gly Val Pro Ser Arg Phe Ser Gly Ser
85 90 95

Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
100 105 110

Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Trp Ser Asn Pro Tyr Thr
115 120 125

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
130 135 140

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
145 150 155 160

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
165 170 175

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
180 185 190

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
195 200 205

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
210 215 220

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
225 230 235 240

Asn Arg Gly Glu Cys
245

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 746 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TTCGAAGAGA TGTCAATGAC TCGTGTGTCC TGGAGTGGTA CCCTACCTCG ACATAGTAGG 60

AGAAGAACCA TCGTTGTCGA TGTCCACAGG TGAGGCTGTA GGTCTACTGG GTCTCGGGTT 120

CGTCGGACTC GCGGTCGCAC CCACTGTCTC ACTGGTAGTG GACATTCCGT TCACTCCTGT 180

ATATATTAGC CAATTGGACC ATGGTCGTCT TCGGTCCATT CCGAGGTTTC GACGACTAGA 240

GACCACGTTG GTCAAACCTT TGACCACACG GTTCGTCTAA GTCGCCATCG CCATCGCCAT 300

GGCTGAAGTG GAAGTGGTAG TCGTCGGAGG TCGGTCTCCT GTAGCGGTGG ATGATGACGG 360

TTGTCATAAC CTCATTAGGC ATGTGCAAGC CGGTTCCCTG GTTCCACCTT TAGTTTGCTT 420

GACACCGACG TGGTAGACAG AAGTAGAAGG GCGGTAGACT ACTCGTCAAC TTTAGACCTT 480

GACGGAGACA ACACACGGAC GACTTATTGA AGATAGGGTC TCTCCGGTTT CATGTCACCT 540

TCCACCTATT GCGGGAGGTT AGCCCATTGA GGGTCCTCTC ACAGTGTCTC GTCCTGTCGT 600

TCCTGTCGTG GATGTCGGAG TCGTCGTGGG ACTGCGACTC GTTTCGTCTG ATGCTCTTTG 660

TGTTTCAGAT GCGGACGCTT CAGTGGGTAG TCCCGGACTC GAGCGGGCAG TGTTTCTCGA 720

AGTTGTCCCC TCTCACAATC TTCGAA 746

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 436 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:3..14

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:18..434

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AA GCT TTA CAG TTA CNC AGC ACA CAG GAC CTC ACC ATG GGA TGG AGC 47
Ala Leu Gln Leu Ser Thr Gln Asp Leu Thr Met Gly Trp Ser
1 5 10

TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGT GTC CAC TCC CAG 95
Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly Val His Ser Gln
15 20 25

```
GTC CAA CTG CAG GAG AGC GGT CCA GGT CTT GTG AGA CCT AGC CAG ACC      143
Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg Pro Ser Gln Thr
            30                  35                  40

CTG AGC CTG ACC TGC ACC GTG TCT GGC TTT TCG TTA ACC AGT TAT GGT      191
Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Ser Leu Thr Ser Tyr Gly
        45                  50                  55

GTC CAC TGG GTG AGA CAG CCA CCT GGA CGA GGT CTT GAG TGG ATT GGA      239
Val His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp Ile Gly
    60                  65                  70

GTG ATG TGG AGA GGT GGA AGC ACA GAC TAC AAT GCA GCT TTC ATG TCC      287
Val Met Trp Arg Gly Gly Ser Thr Asp Tyr Asn Ala Ala Phe Met Ser
75                  80                  85                  90

AGA CTG AAC ATC ACC AAG GAC AAC AGC AAG AAC CAG GTG AGC TTA AGA      335
Arg Leu Asn Ile Thr Lys Asp Asn Ser Lys Asn Gln Val Ser Leu Arg
                95                  100                 105

CTC AGC AGC GTG ACA GCC GCC GAC ACC GCG GTC TAT TAT TGT GCA AAA      383
Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys Ala Lys
            110                 115                 120

TCC ATG ATA ACG ACC GGC TTC GTG ATG GAC TCC TGG GGT CAA GGC TCA      431
Ser Met Ile Thr Thr Gly Phe Val Met Asp Ser Trp Gly Gln Gly Ser
        125                 130                 135

CTA GT                                                              436
Leu
```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Ala Leu Gln Leu
1

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 139 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Ser Thr Gln Asp Leu Thr Met Gly Trp Ser Cys Ile Ile Leu Phe Leu
1 5 10 15

Val Ala Thr Ala Thr Gly Val His Ser Gln Val Gln Leu Gln Glu Ser
20 25 30

Gly Pro Gly Leu Val Arg Pro Ser Gln Thr Leu Ser Leu Thr Cys Thr
35 40 45

Val Ser Gly Phe Ser Leu Thr Ser Tyr Gly Val His Trp Val Arg Gln
50 55 60

Pro Pro Gly Arg Gly Leu Glu Trp Ile Gly Val Met Trp Arg Gly Gly

55

65 70 75 80

Ser Thr Asp Tyr Asn Ala Ala Phe Met Ser Arg Leu Asn Ile Thr Lys
85 90 95

Asp Asn Ser Lys Asn Gln Val Ser Leu Arg Leu Ser Ser Val Thr Ala
100 105 110

Ala Asp Thr Ala Val Tyr Tyr Cys Ala Lys Ser Met Ile Thr Thr Gly
115 120 125

Phe Val Met Asp Ser Trp Gly Gln Gly Ser Leu
130 135

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 436 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TTCGAAATGT CAATGNGTCG TGTGTCCTGG AGTGGTACCC TACCTCGACA TAGTAGGAGA 60

AGAACCATCG TTGTCGATGT CCACAGGTGA GGGTCCAGGT TGACGTCCTC TCGCCAGGTC 120

CAGAACACTC TGGATCGGTC TGGGACTCGG ACTGGACGTG GCACAGACCG AAAAGCAATT 180

GGTCAATACC ACAGGTGACC CACTCTGTCG GTGGACCTGC TCCAGAACTC ACCTAACCTC 240

ACTACACCTC TCCACCTTCG TGTCTGATGT TACGTCGAAA GTACAGGTCT GACTTGTAGT 300

GGTTCCTGTT GTCGTTCTTG GTCCACTCGA ATTCTGAGTC GTCGCACTGT CGGCGGCTGT 360

GGCGCCAGAT AATAACACGT TTTAGGTACT ATTGCTGGCC GAAGCACTAC CTGAGGACCC 420

CAGTTCCGAG TGATCA 436

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GACATTCAGC TGACCCAGTC TCCA 24

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GATCAAGCTT GACATTCAGC TGACCCAGTC TCCA 34

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ACTAGTCGAC CATCCTCCTT TTCTGTTTCT CTAGGAG 37

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GATCAAGCTT CTCTACAGTT ACTGAGCACA 30

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CCGATTATAT ATGTCCTCAC TTGCCTTACA GGTGATGGTC AC 42

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AGTGAGGACA TATATAATCG GTTAACCTGG TACCAGCAGA AG 42

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AGTTTCCAAA CTGGTTGCAC CAGAGATCAG CAGCTTTGG 39

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GGTGCAACCA GTTTGGAAAC TGGTGTGCCA AGCAGA 36

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GTACGGATTA CTCCAATACT GTTGGCAGTA GTAGGTGGC 39

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CAGTATTGGA GTAATCCGTA CACGTTCGGC CAAGGGACC 39

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GATCAAGCTT CTAACACTCT CCCCTGTTGA 30

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GATCAAGCTT TACAGTTACT CAGCACACAG 30

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GTGGACACCA TAACTGGTGA AGGTGAAGCC 30

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

AGTTATGGTG TCCACTGGGT GAGACAGCCA 30

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

TTGTAGTCTG TGCTTCCACC TCTCCACATC ACTCCAATCC ACTCAAG 47

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GGAGTCCATC ACGAAGCCGG TCGTTATCAT GGATTTTGCA CAATAATAGA C 51

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

AAATCCATGA TAACGACCGG CTTCGTGATG GACTCCTGGG GTCAAGGCTC ACTAGTCACA 60

GTCTCCTCAG CC 72

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

TAGAGTCCTG AGGGAATTCG GACAGCCGGG AAGGTG 36

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GTTGTCCTTG GTGATGTTCA GTCTGGACAT GAAAGCTGC 39

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

CTGAACATCA CCAAGGACAA CAGCAAGAAC CAGTTCAGC 39

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

ACTGGTTAAC GAAAAGCCAG ACACGGTGCA GGTCAG 36

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

GGCTTTTCGT TAACCAGTTA TGGTGTCCAC TGGGTG 36

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

AAATTGCCGT TTCGAAGTGT CTACCAGCAT TGTCAC 36

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

AAATTGCCGT TTCGAATTGT CCTTGGTGAT GTTCAG 36

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

TTCGAAACGG CAATTTAGCT TGAGACTCAG CAGC 34

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

GAAGCACAGA CTACAATGCA GCTTTCATGT CCAGAGTGAC AATGCTG 47

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

GATCAAGCTT TACAGTTACT CAGCACAG 28

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

ACTGGTTAAC GAAAAGCCAG ACACGGTGCA GGTCAG 36

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

GGCTTTTCGT TAACCAGTTA TGGTGTCCAC TGGGTG 36

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

TAGAGTCCTG AGGGAATTCG GACAGCCGGG AAGGTG 36

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

AACCAGGTGA GCTTAAGACT CAGCAGCGTG ACA 33

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

TCTTAAGCTC ACCTGGTTCT TGCTGTTGTC CTT 33

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

Ser Tyr Gly Val His
1 5

CLAIMS:

1. A monoclonal antibody having donor CDRs of foreign origin and a recipient framework region having a sequence of human or primate origin, wherein the original amino acid residue in position 29 or 78 of the sequence of the recipient framework region of the heavy chain is replaced by a replacement amino acid residue that is the same or similar to that in the corresponding position of the sequence of the corresponding framework region of the heavy chain of the antibody from which the CDRs are derived.

2. A monoclonal antibody according to claim 1, wherein the original amino acid residues in both positions 29 and 78 of the sequence of the recipient framework region of the heavy chain are replaced by replacement amino acids that are the same or similar to the amino acids in the corresponding positions of the corresponding framework region of the antibody from which the CDRs are derived.

3. A monoclonal antibody according to claim 1 or 2, wherein one or both of the original amino acid residues of the recipient framework region are replaced by a replacement amino acid residues of similar size, hydrophobicity and charge to the amino acids in the corresponding positions of the corresponding framework region of the antibody from which the CDRs are derived.

4. A monoclonal antibody according to any of the preceding claims, wherein the original amino acid residues of the recipient framework region are the same or different and are tyrosine, histidine, tryptophan or 2-phenyl-alanine.

5. A monoclonal antibody according to claim 4, wherein the replacement amino acid residues are the same or different and are selected from glycine, alanine, valine, serine or leucine.

6. A monoclonal antibody according to any of the preceding claims wherein the recipient framework region is from a heavy chain selected from LES-C, T52, Ab44, HIGI and NEW.

7. A monoclonal antibody according to any of the preceding claims, wherein the CDRs are of rat, mouse rabbit, or hamster origin.

8. A monoclonal antibody according to any of the preceding claims, wherein the heavy chain of the antibody from which the CDRs are derived is a murine heavy chain in Kabat groups IB and IIC.

9. A monoclonal antibody according to any of the preceding claims wherein the antibody binds to CD38.

10. A monoclonal antibody according to claim 9 having a nucleotide sequence as shown in figures 3, 3a and 4.

11. A monoclonal antibody according to any of the preceding claims, wherein the donor CDR is CDRHI.

12. A monoclonal antibody according to claim 11, wherein CDRHI has a sequence of SYGVH.

13. A method of producing an antibody according to any of the above claims comprising the steps of:

(i) obtaining the sequence of a donor heavy chain;

(ii) selecting a recipient human or primate framework by best-fit homology method;

(iii) replacing the amino acid residue in position 29 or 78 of the sequence of the recipient framework region of the heavy chain by an amino acid that is the same or similar to that in the corresponding position of the sequence of the corresponding framework region of the antibody from which the CDRs are derived;

(iv) grafting donor CDRs into the recipient human framework.

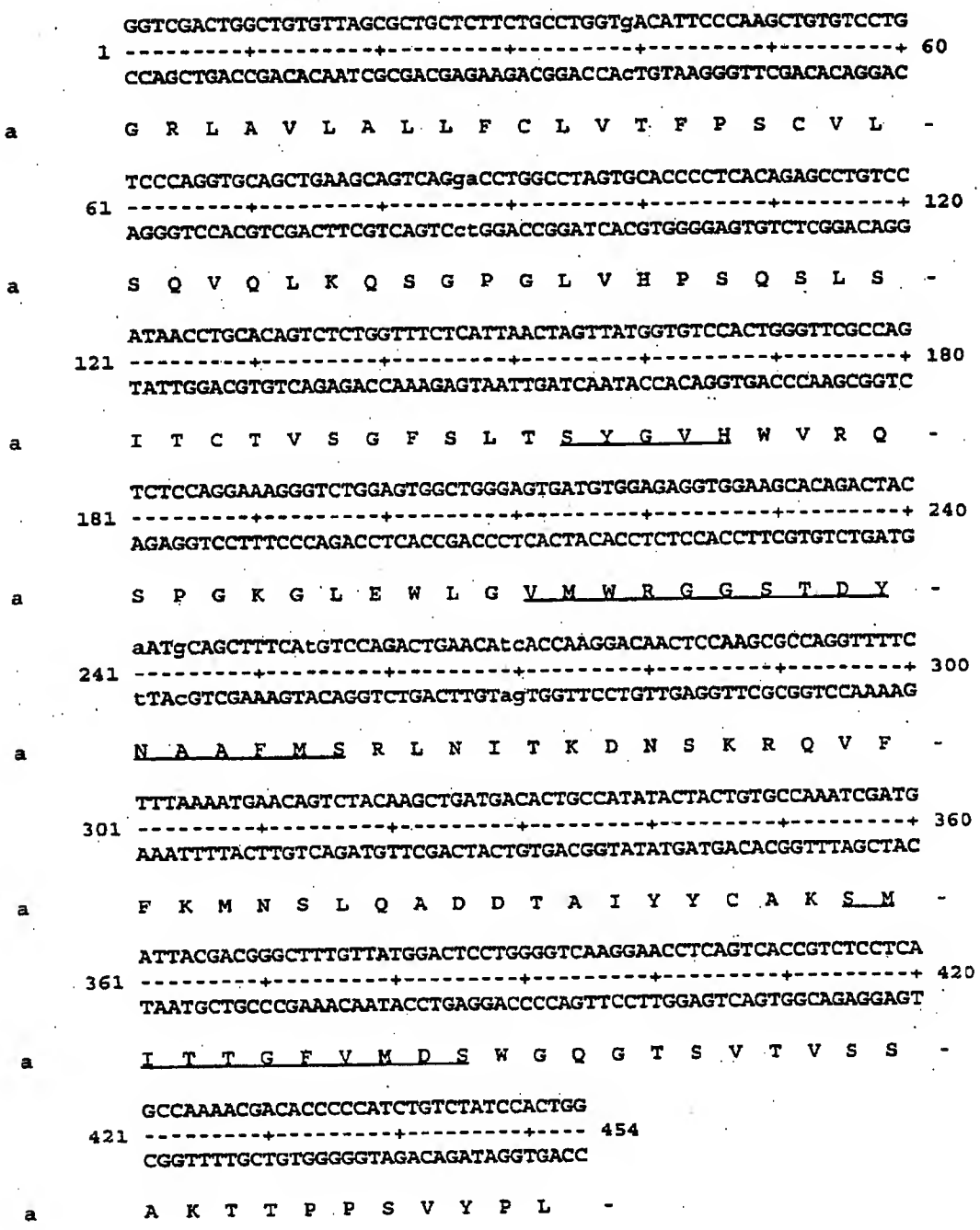
14. Use of an antibody according to any of the preceding claims for the treatment of cancer and autoimmune diseases.

15. Use of an antibody according to claim 9 or 10 for treatment of multiple myeloma, lymphoma and autoimmune diseases such as rheumatoid arthritis.

16. Use of an antibody according to any of claims 1 to 12 for the manufacture of a medicament for the treatment of cancer or an autoimmune disease.

17. Use of an antibody according to any of claims 1 to 12 for the manufacture of a medicament for the treatment of multiple myeloma, lymphoma, or rheumatoid arthritis.

18. A pharmaceutical composition comprising an antibody according to any of claims 1 to 12 and a physiologically acceptable diluent or carrier.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61 120
AGGGTCCACGTCGACTTCGTCAGTCctGGACCGGATCACGTGGGGAGTGTCTCGGACAGG
a S Q V Q L K Q S G P G L V H P S Q S L S -
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121 180
TATTGGACGTGTCAGAGACCAAAGAGTAATTGATCAATACCACAGGTGACCCAAGCGGTC
a I T C T V S G F S L T S Y G V H W V R Q -
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181 240
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241 300
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301 360
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361 420
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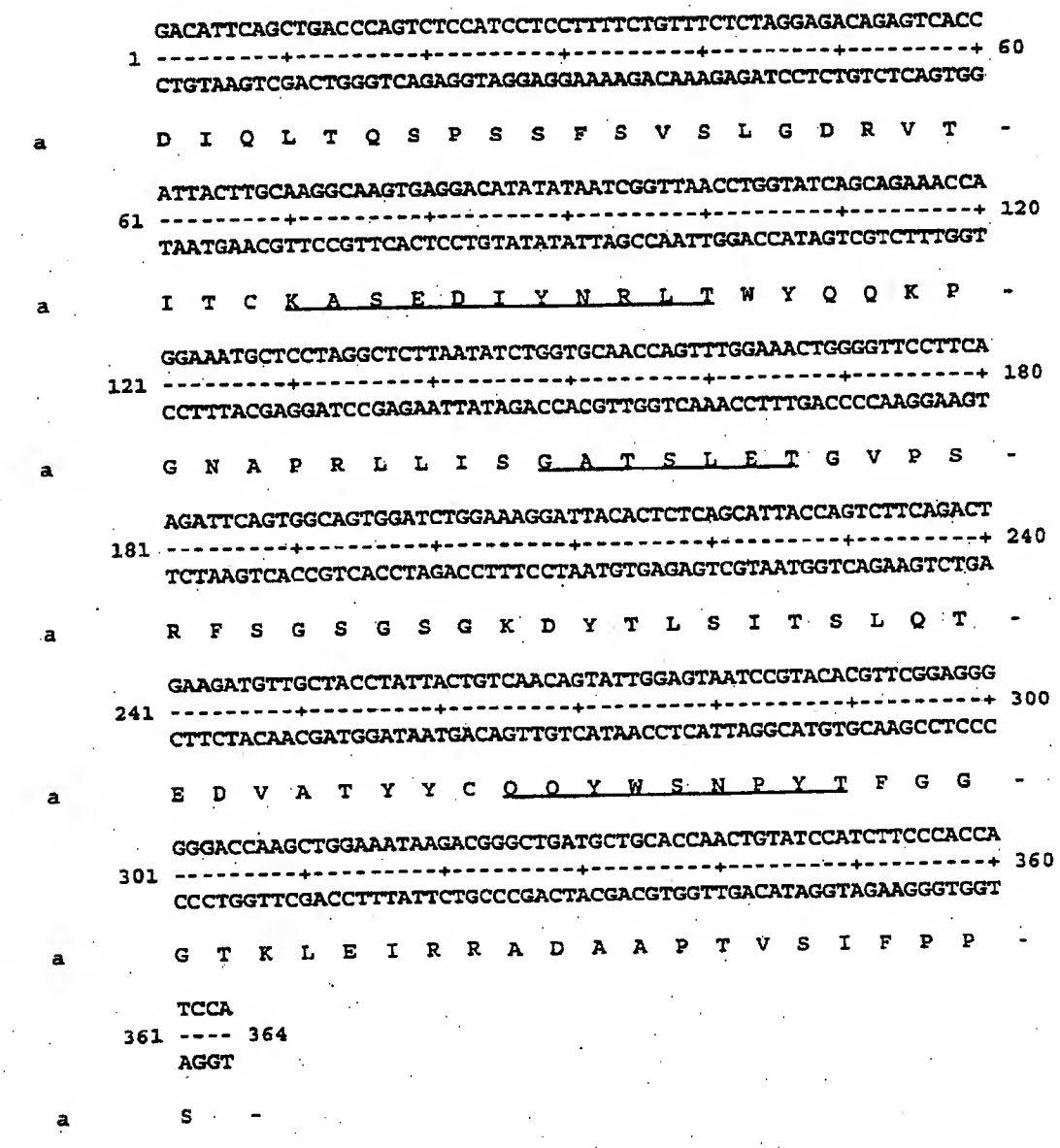
Fig. 1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61 120
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121 180
CCTTTACGAGGATCCGAGAATTATAGACCACGTTGGTCAAACCTTTGACCCCAAGGAAGT
a G N A P R L L I S G A T S L E T G V P S -
AGATTCAGTGGCAGTGGATCTGGAAAGGATTACACTCTCAGCATTACCAGTCTTCAGACT
181 240
TCTAAGTCACCGTCACCTAGACCTTTCCTAATGTGAGAGTCGTAATGGTCAGAAGTCTGA
a R F S G S G S G K D Y T L S I T S L Q T -
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241 300
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a E D V A T Y Y C Q Q Y W S N P Y T F G G -
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301 360
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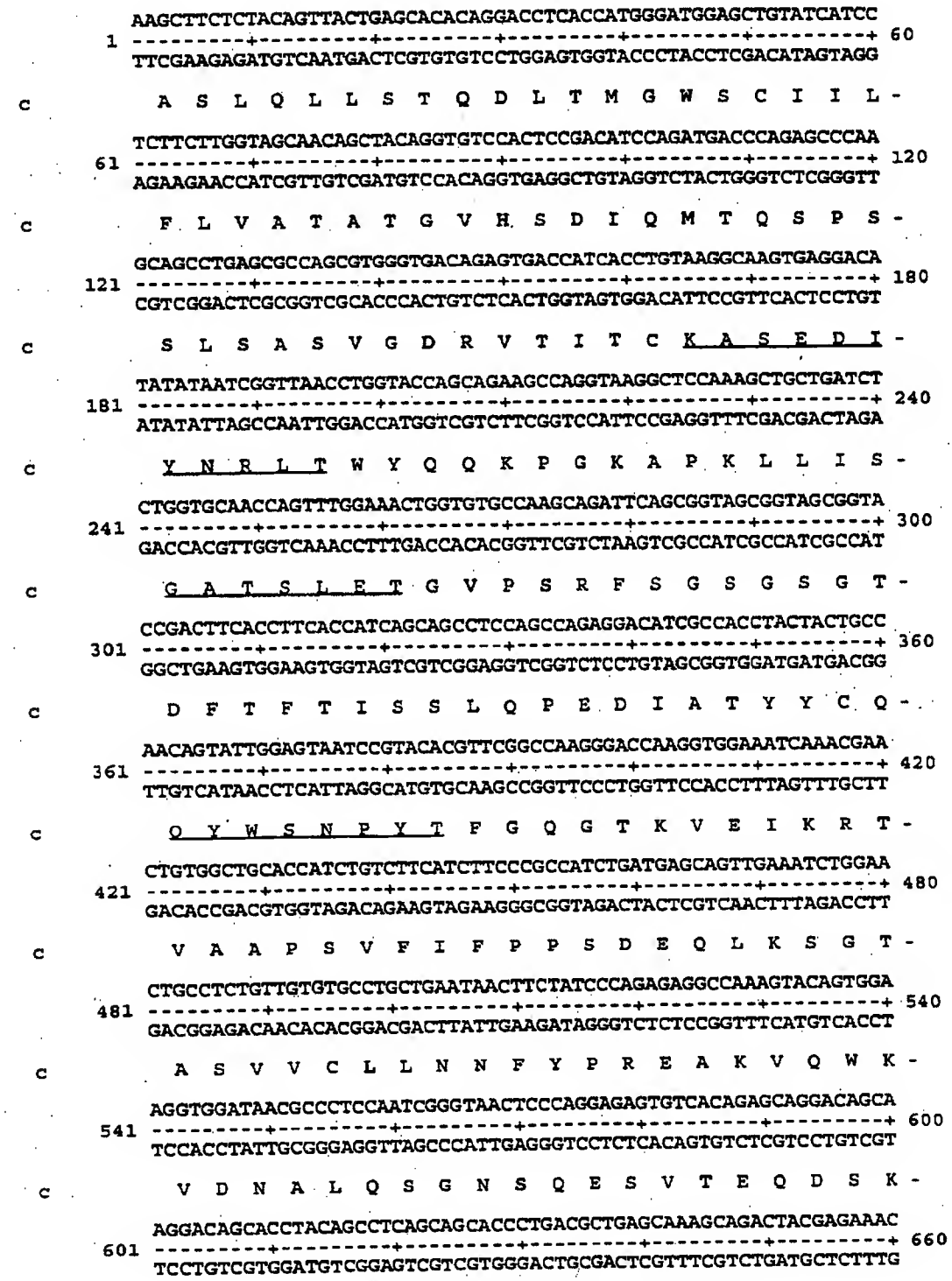
361 364
AGGT
a S -

Fig. 2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120
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c F L V A T A T G V H S D I Q M T Q S P S -
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121
180
CGTCGGACTCGCGGTCGCACCCACTGTCTCACTGGTAGTGGACATTCCGTTCACTCCTGT
c S L S A S V G D R V T I T C K A S E D I -
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181
240
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241
300
GACCACGTTGGTCAAACCTTTGACCACACGGTTCGTCTAAGTCGCCATCGCCATCGCCAT
c G A T S L E T G V P S R F S G S G S G T -
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301
360
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361
420
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421
480
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481
540
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c A S V V C L L N N F Y P R E A K V Q W K -
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541
600
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660
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Fig. 3

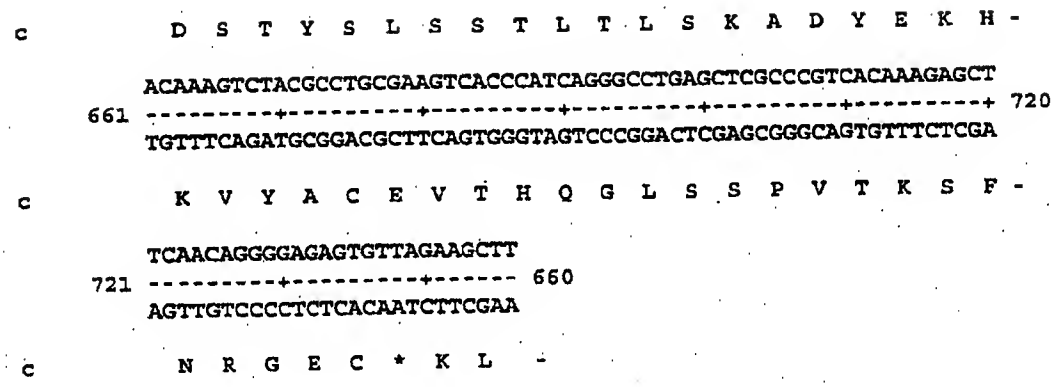
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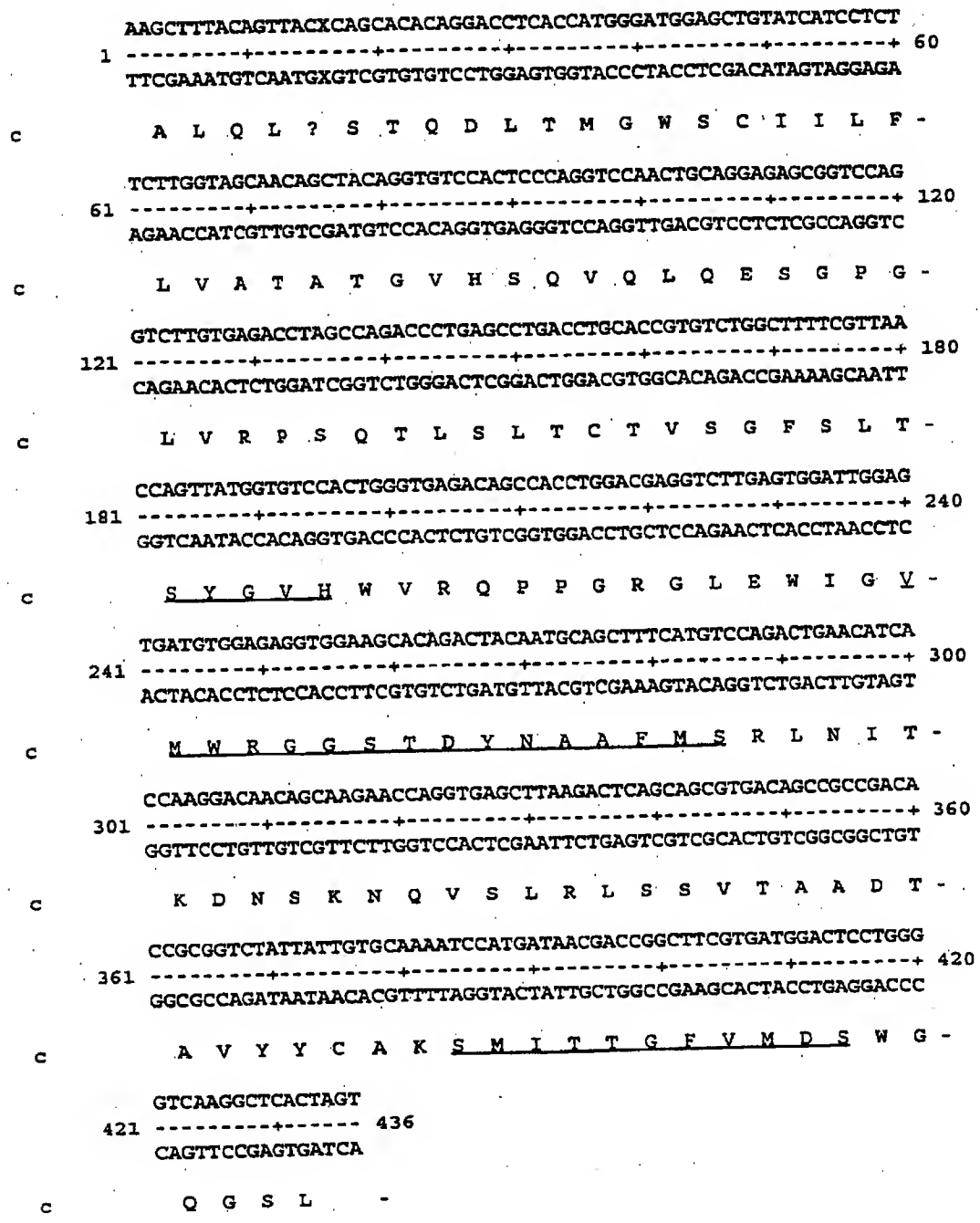
Fig.3a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181 240
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301 360
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Fig. 4

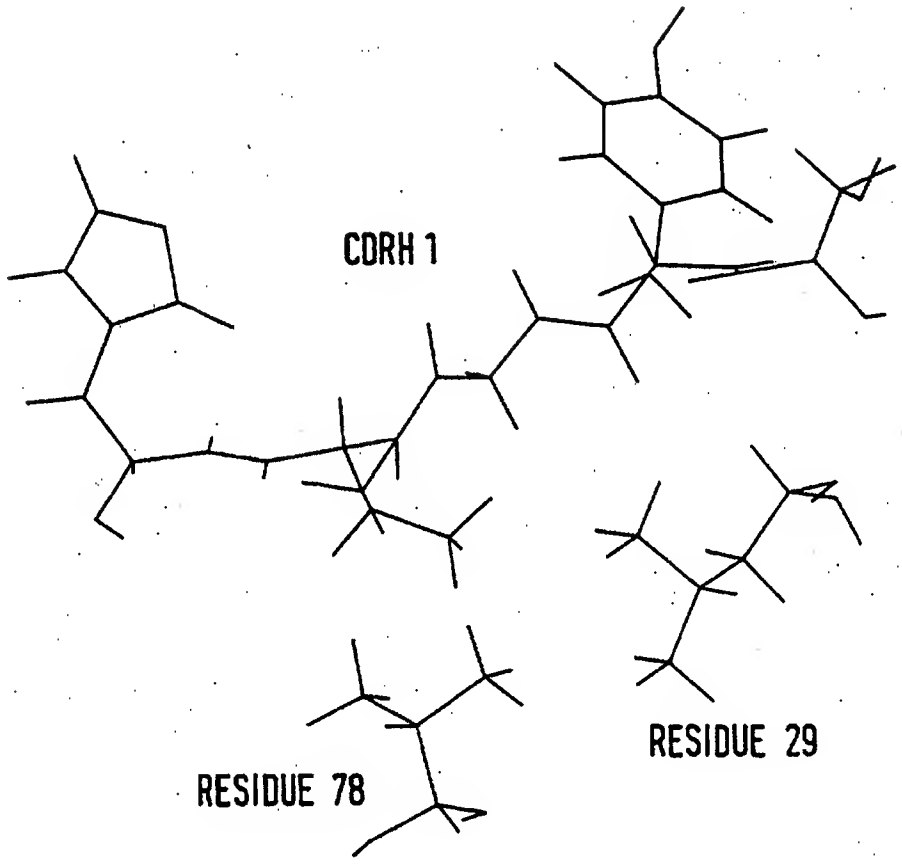

Fig.5

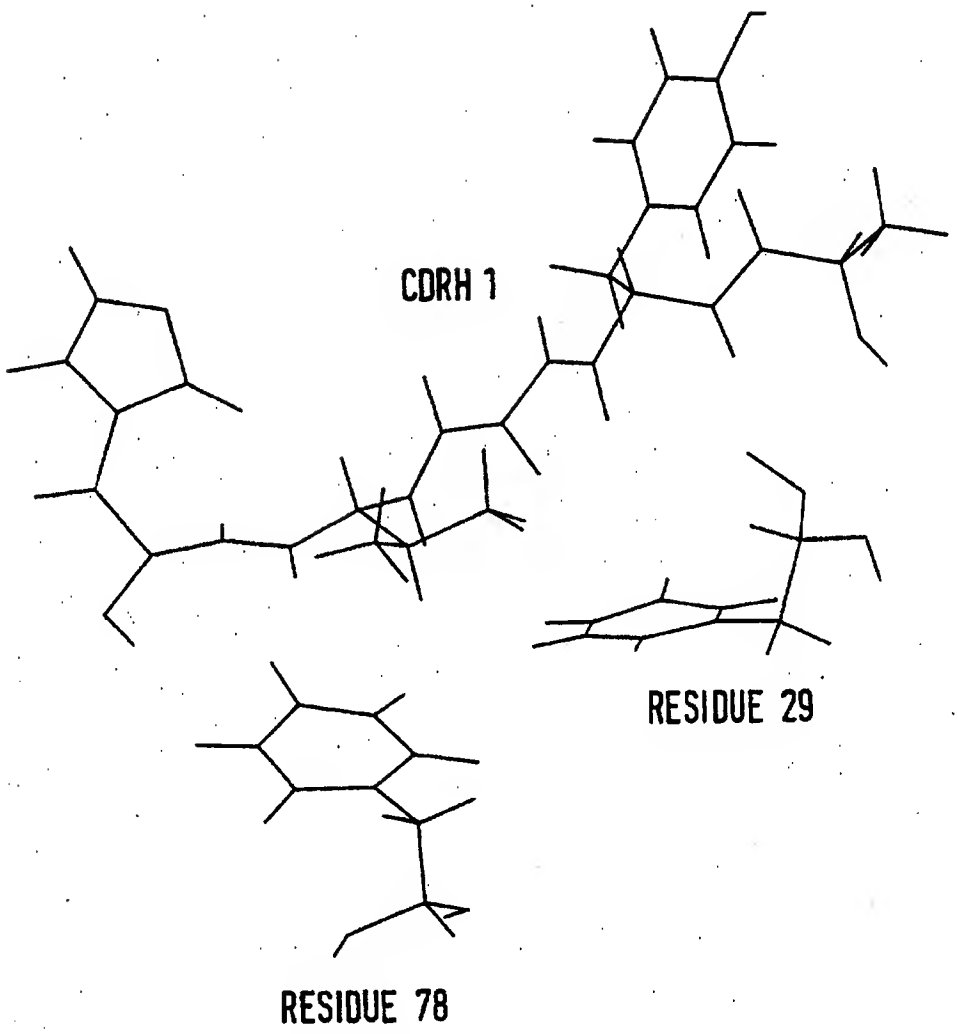

Fig. 6

EFFECT OF VARIOUS HEAVY CHAIN FRAMEWORK SUBSTITUTIONS ON RELATIVE BINDING AFFINITY OF ANT-CD38 ANTIBODIES

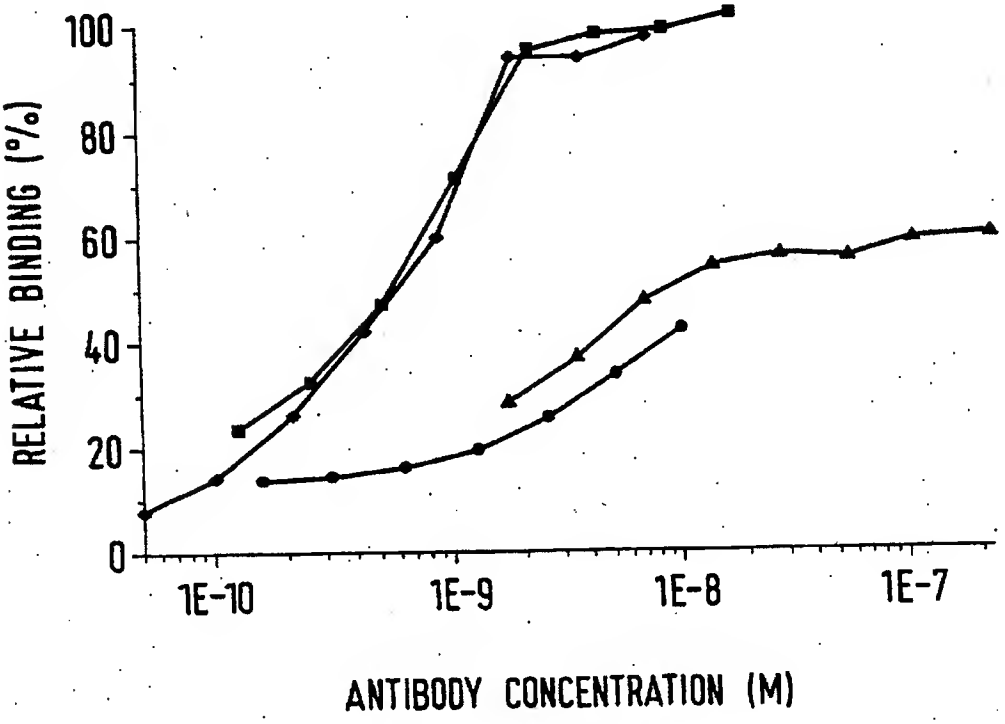

Fig. 7

EFFECT OF VARIOUS HEAVY CHAIN FRAMEWORK SUBSTITUTIONS ON ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY MEDICATED BY CD38 ANTIBODIES

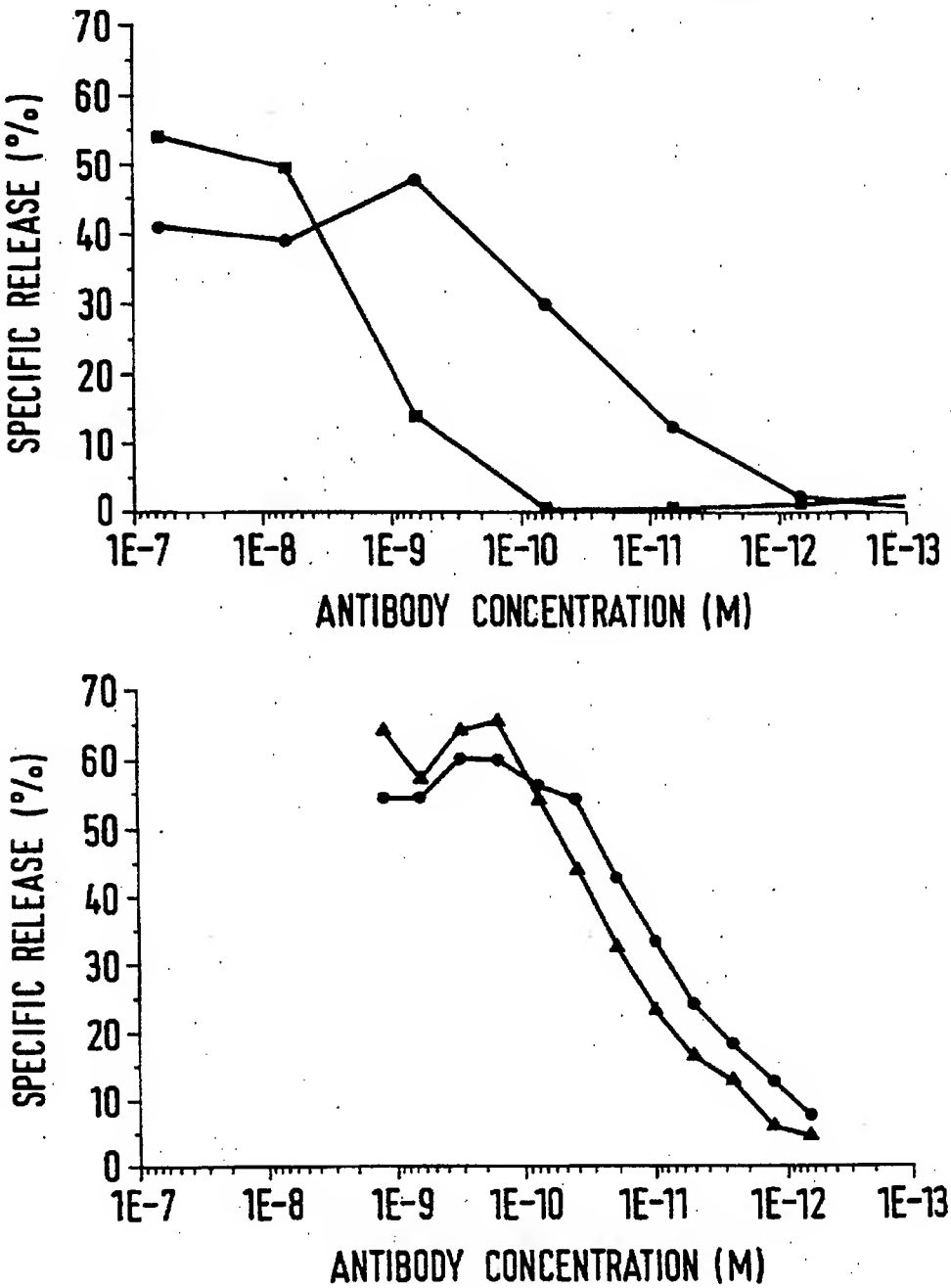

Fig. 8

INTERNATIONAL SEARCH REPORT

Inte· onal Application No
PCT/GB 95/02777

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/46 C07K16/28 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF MOLECULAR BIOLOGY, vol. 235, no. 1, 7 January 1994 LONDON, GB, pages 53-60, XP 000564648 A. CORTI ET AL. 'Idiotope determining regions of a mouse monoclonal antibody and its humanized versions.'	1-5,7
Y	see the whole document	9
Y	WO,A,94 17184 (SCHERING CORPORATION ET AL.) 4 August 1994 see examples see claims	9

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 March 1996	23.04.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/GB 95/02777

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 13805 (CELLTECH LTD.) 23 June 1994 see page 12, line 19 - page 13, line 5 see example 1 see claims	1,3,4,7, 8,13,14, 16,18
X	WO,A,91 09967 (CELLTECH LTD.) 11 July 1991 see table 2 see examples see claims	1,3,5,7, 13-18
X	WO,A,94 09136 (KETTOCK LODGE, CAMPUS 2) 28 April 1994 see claims	1,3,5,7, 13
X	JOURNAL OF MOLECULAR BIOLOGY, vol. 224, no. 2, 20 March 1992 LONDON, GB, pages 487-499, XP 000564649 J. FOOTE ET AL. 'Antibody framework residues affecting the conformation of the hypervariable loops.' see abstract see table 2	1,3,5-7, 11,13
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 89, no. 10, 15 May 1992 WASHINGTON, DC, USA, pages 4285-4289, XP 000275844 P. CARTER ET AL. 'Humanization of an anti-p185HER2 antibody for human cancer therapy.' see abstract see table 1	1,3,5,7, 13-18
A	EP,A,0 481 790 (THE WELLCOME FOUNDATION) 22 April 1992 see claims	1-18
P,X	THE JOURNAL OF IMMUNOLOGY, vol. 155, no. 2, 15 July 1995 BALTIMORE, MD, USA, pages 925-937, J. ELLIS ET AL. 'Engineered anti-CD38 monoclonal antibodies for immunotherapy of multiple myeloma.' see the whole document	1-18

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/02777

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,O, X	IMMUNOLOGY, vol. 83, suppl. 1, 5 - 7 December 1994 OXFORD, GB, page 70 XP 000565931 J. ELLIS ET AL. 'Characterisation of a humanised monoclonal anti-CD38 antibody.' see abstract	1-18

1

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/GB95/02777

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 14,15
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 14 and 15 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

.nformation on patent family members

Inte ·nal Application No
PCT/GB 95/02777

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9409136		EP-A-	0664834	02-08-95
		GB-A-	2286399	16-08-95
EP-A-481790	22-04-92	AU-B-	645355	13-01-94
		AU-B-	8591491	30-04-92
		CA-A-	2053585	18-04-92
		JP-A-	6090752	05-04-94
		NZ-A-	240249	25-03-94
		ZA-A-	9108248	16-04-93